Investigation of Novel Circuits Involved in Virgin Female Receptivity of *Drosophila melanogaster*

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Resumo

Corte é um comportamento que permite a exibição do fitness de um indivíduo para o sexo oposto e, consequentemente possibilita o acasalamento. Este comportamento é essencial para a reprodução e contém uma forte componente inata em todos os animais. A corte em *Drosophila melanogaster* consiste numa série de ações estereotipadas executadas pelo macho para a fêmea. Ele canta, toca e lambe o abdómen da fêmea, enquanto ela avalia a informação transmitida pelo macho.

O nosso objetivo foi contribuir para um melhor conhecimento dos circuitos neurais que medeiam o comportamento da fêmea durante a corte do macho, especificamente a sua decisão sobre aceitar ou rejeitar o macho, a sua recetividade. Usando um paradigma comportamental específico para estudar a recetividade da fêmea e um inibidor neuronal ativado por temperatura, investigamos onze linhas GAL4 para o efeito do silenciamento neuronal na recetividade da fêmea.

Descobrimos que o silenciamento dos neurónios *apterous-GAL4* reduz a recetividade. Numa série de experiências controlo, verificámos que este fenótipo não é um efeito secundário devido a cegueira, redução da atratividade, locomoção comprometida ou ausência de Apterous na população adulta. A anatomia de *apterous-GAL4* sugere que não existe inibição de neurónios envolvidos na fase inicial do processamento olfatório e auditório. Do mesmo modo, os neurónios sensoriais ativados pelo péptido do sexo e responsáveis pelo “postmating switch” (sex peptide sensory neurons)

também não estão silenciados, indicando que os neurónios *apterous* intervêm na decisão das fêmeas virgens.

Adicionalmente, o silenciamento dos neurónios *apterous* resulta em mais dois fenótipos: uma redução na ovulação e uma probóscide permanentemente estendida. Recorrendo a uma estratégia interseccional usando treze linhas GAL80, identificamos dois grupos de neurónios. No primeiro grupo, a expressão de GAL80 recupera o fenótipo de *apterous-GAL4* na recetividade, ovulação e extensão da probóscide. Este grupo de neurónios sintetiza Colina-acetiltransferase, Fruitless e Leucokinin. O segundo grupo de neurónios, possivelmente um subconjunto do primeiro grupo, é caracterizado por recuperar unicamente a recetividade (embora parcialmente). Este segundo conjunto de neurónios expressa Tirosina hidroxilase, Glutamato descarboxilase e Cryptochrome.

Com o objetivo de localizar estes neurónios investigamos diferenças na expressão. Comparamos diretamente os perfis de expressão através do método “brain alignment”. Contudo, não fomos capazes de identificar de forma inequívoca a sua localização, o que sugere que o conjunto de neurónios sob investigação é pequeno.

Enquanto a maior parte da literatura centra-se no controlo neuronal do “postmating switch”, este trabalho revela importantes informações para futuros estudos focados na recetividade da fêmea virgem. Os resultados obtidos neste estudo apontam para um pequeno e novo grupo de neurónios centrais que medeiam a decisão da fêmea virgem sobre aceitar ou não o macho.

Summary

Courtship is a behavior that allows the display of fitness of one sex to the other and gates possible subsequent mating. This behavior is crucial for reproduction and has strong innate components in all animals. Courtship in *Drosophila melanogaster* consists of a series of highly stereotyped actions that the male performs towards the female. He sings with vibrations of the wings, touches and licks her abdomen, while she evaluates the information presented to her.

We aimed at contributing to the understanding of the neural circuitry that mediates female behavior during courtship, more specifically her choice whether to accept a mate or not, her receptivity. Employing an established single-pair receptivity assay and a temperature-inducible neuronal inhibitor, we screened eleven GAL4-lines for the effect of silenced neurons on receptivity.

We uncovered that silencing *apterous-GAL4* labeled neurons reduces receptivity. In a series of control experiments we ascertained that this phenotype is not a side-effect of blindness, diminished attractiveness, compromised locomotion or the absence of Apterous in the adult. Furthermore, *apterous-GAL4* anatomy suggests that we are not inhibiting neurons involved in the early stages of olfaction or audition. Likewise we do not silence sex peptide sensory neurons triggering the postmating switch, indicating that *apterous* neurons mediate the choice of virgin females.

Silencing *apterous* neurons results in two further phenotypes: reduced egg-laying and a constitutively extended proboscis and bloatedness. We employed an intersectional strategy screening

thirteen GAL80-lines and identified two sets of neurons. In the first set GAL80-expression rescues *apterous-GAL4*-mediated receptivity, egg-laying and the proboscis extension/bloatedness-phenotype. These neurons are *choline acetyltransferase*, *fruitless* and *leucokinin* positive. The second set of neurons, possibly a subset of the first, is characterized by rescuing receptivity alone (albeit partially). This second set is additionally *tyrosine hydroxylase*, *glutamic acid decarboxylase* and *cryptochrome* positive.

In order to locate these neurons we searched for differences in expression between the *apterous* expression pattern and the *apterous* expression pattern minus the intersection. For this we employed the technique “brain alignment”. However, we were not able to unambiguously identify the location of neurons forming an intersection, suggesting that the sought set of neurons is small.

While a majority of the literature addresses the neuronal control of the postmating switch, this work provides valuable information for follow-up studies investigating virgin receptivity. The results obtained in this work point to a small novel set of central neurons that mediate the decision of virgin females whether to accept a mate or not.

Contents

Abbreviations List

abg	abdominal ganglion
AL	antennal lobe
AMMC	antennal mechanosensory and motor center
CHC	cuticular hydrocarbon
Com. ov.	Common oviduct
dsx	*doublesex*
FLA	flange
FLP	flippase
FRT	flippase recognition target
fru	*fruitless*
GAL4	yeast transcription activator protein GAL4
GAL80	yeast negative regulatory protein GAL80
GNG	gnathal ganglia
GR	gustatory receptor
GRN	gustatory receptor neuron
JH	juvenile hormone
JO	Johnston’s organ
KC	Kenyon cells
Kir2.1	inwardly rectifying potassium channel 2.1
LH	lateral horn
MB	mushroom body

OL	optic lobe
OTU	optic tubercle
OR	odorant receptor
ORN	olfactory receptor neuron
PENP	periesophageal neuropils
PMR	postmating response
PRW	prow
RNAi	RNA interference
SAD	saddle
SEZ	subesophageal zone
SFP	seminal fluid proteins
SOG/SEG[1]	sub(o)esophageal ganglion
SP	sex peptide
UAS	upstream activating sequence

[1] Esophagus (American English), Oesophagus (British English); SOG however, is used predominantly throughout the *Drosophila* literature.

Recently, Ito et al. (2014) have tried to clean up ambiguities regarding the nomenclature of the insect brain. The triangular shaped neuropil below the esophagus is usually referred to as SOG. Ito et al. recommend using the term SEZ for subesophageal zone, which comprises the gnathal ganglia (GNG) and the periesophageal neuropils (PENP), i.e. the prow (PRW), the flange (FLA), the saddle (SAD) and the antennal mechanosensory and motor center (AMMC). The SOG on the other hand consist of the gnathal ganglia (GNG) and only parts of the periesophageal neuropils (PENP), i.e. the prow (PRW), the flange (FLA) and the most medial parts of the saddle (SAD). For the sake of simplicity we will use the term SOG for the area just described.

VNC ventral nerve cord

I. Introduction

When *Drosophila melanogaster* flies of both sexes meet, courtship will commence. This is an innate behavior – a behavior that is triggered by certain sensorial cues without ever having experienced them before. It is mediated by neural circuits that were shaped throughout evolution. This means that the complex interplay of genes that defines an organism of a given species, also defines how certain sets of neurons connect. Those neural networks will be almost identical across all individuals of the species (reviewed in Hall 1994 and Spieth 1974; Manoli, Meissner, and Baker 2006). Examples of innate behaviors are phototaxis and odortaxis: flies will walk toward light as well as towards an attractive odor source. They will initiate feeding when they taste sweet, tend to walk against the force of gravitation and hold tight to the ground they are walking on when airborne turbulences threaten to blow them away uncontrollably. Reliable execution of any of these behaviors is crucial for the survival of the animal (Benzer, 1971; Kamikouchi, 2013; Spieth, 1974).

One of the big goals in neuroscience is to understand how the structure of neural networks relates to their function, in the process of transforming sensorial input into behavioral output. Hard-wired circuits in simple organisms can be helpful in this pursuit. Innate behaviors are elicited in naïve animals in situations they never encountered before by circuits that are genetically defined to perform a specific behavior. On the contrary learned behaviors are developed in response to experience by modification of existing circuits. Innate behaviors have the advantage of being virtually identical from one specimen to the next and thus allow understanding of the relationship

between behavior, its neural substrate and the genes that build it at a resolution that can reach down to a single neuron. Findings in the circuits of innate behaviors in *Drosophila* will allow the elucidation of widespread basic principles, and even if they may not be directly translatable into human or even mammalian brain function, they will likely inspire new insights (Baker, Taylor, & Hall, 2001; Manoli et al., 2006).

This study focuses on female behavior of *Drosophila melanogaster* during courtship. It aims at identifying neurons involved in female receptivity behavior and thus contributing to the understanding of the neural processes that are responsible for the female's decision whether to accept a male for copulation or not.

Courtship in Drosophila – Behavioral Description

Placed into a small observation chamber, soon after perceiving a female, the male will orient towards her and follow her. A mature virgin will then speed off, occasionally pause, and decamp again. He will follow her and describe quick half circles around her. While courting he will always be oriented towards her, which may be interrupted by short phases of other behaviors. During this interleaved succession of chasing and circling he will occasionally tap her with a foreleg and vigorously sing a courtship song, also called "love song" (Bastock & Manning, 1955; Dickson, 2008; Spieth, 1974). He does this by extending one of his wings orthogonally to his body and vibrating it rapidly, thus creating sound waves of defined frequencies and amplitudes. This may go on for a few seconds or many minutes; eventually he will lick her genitalia and bend his abdomen forward in the attempt to join genitalia and copulate successfully (Bastock &

Manning, 1955; Spieth, 1974). If the female is receptive she will open her vaginal plates and thereby allow copulation (Connolly & Cook, 1973; L Tompkins & Hall, 1983). Her part in courtship is obviously subtle but most likely as crucial as his. A detailed description of the female behavior during courtship was performed by Lasbleiz et al. (2006). This study however lacks a strong mechanistic component and the behaviors to be annotated were defined manually, thus potentially missing patterns that could be uncovered by completely automated analysis. This and other studies indicate that her movement, the pausing and decamping are possibly integral components of a quality courtship giving him valuable cues of how to respond next (Bussell, Yapici, Zhang, Dickson, & Vosshall, 2014; Laurie Tompkins, Gross, Hall, Gailey, & Siegel, 1982; Trott, Donelson, Griffith, & Ejima, 2012). During a courtship sequence that culminates in copulation, the female might have fended him off with her legs, flicked both her wings at him and extruded her ovipositor towards the male; Gestures that have been tentatively interpreted as coyness of generally receptive females (Spieth, 1974). Likewise they may serve as honest rejection behaviors as recently mated females will display rejection behaviors with greater vigor and frequency (Connolly and Cook 1973; reviewed in Lasbleiz, Ferveur, and Everaerts, 2006). Licking of the females genitalia by the male is often followed by copulation attempts (J. Hall, 1994), which may be promoted by secretion of a tiny droplet from the abdomen of the female (Lasbleiz et al., 2006). Copulation then triggers striking changes in the female's behavior; she will drastically increase her egg-laying and reduce her receptivity. These changes are referred to as postmating responses (PMRs) or as result of the postmating switch (Bastock & Manning, 1955; Yapici, Kim, Ribeiro, & Dickson, 2008).

Courtship in Drosophila – Description of the Genetic Underpinnings

The described differences in behavior between male and female constitute clear behavioral dimorphisms. How do they relate to neural dimorphisms? Early attempts to answer this question used flies that were part male and part female – so called sex mosaic flies or gynandromorphs (J. Ferveur & Greenspan, 1998; J. Hall, 1977, 1979; Hotta & Benzer, 1976; Schilcher & Hall, 1979; Szabad & Fajszi, 1982; L Tompkins & Hall, 1983), reviewed in (Baker et al., 2001). These studies were able to imply roughly defined areas of the brain and ventral nerve cord (VNC) in courtship related, sex-specific behaviors, by showing that the respective tissue had to be male or female for the fly to be able to perform certain behaviors; for instance they mapped female receptivity to a bilateral cluster in the dorsal protocerebrum (Szabad & Fajszi, 1982; L Tompkins & Hall, 1983).

A mutation created by X-ray mutagenesis in 1963 (Gailey and Hall 1989; J. C. Hall 1978) gained enduring attention of *Drosophila* scientists. This mutation resulted in sterile males due to abnormal courtship behavior and was therefore called *fruitless (fru)*. Males were unable to bend their abdomen and thus could not copulate (J. C. Hall, 1978). They courted other males as well as females and stimulated other males to court them (J. C. Hall, 1978). Females appeared unaffected by this mutation. These phenotypes of *fruitless* mutants were only observed when fru^M was affected, the male-specific product of the fruitless locus (Baker et al., 2001; Demir & Dickson, 2005; Ito & Fujitani, 1996; Ryner et al., 1996). In contrast to other *fruitless* isoforms that are common to males and females, Fru^M is a male-specific transcription factor, which is expressed only in neurons -

several of which have been shown to be crucial for reproductive behaviors. The protein is not expressed in the female due to a remaining stop codon in the mRNA molecule as a consequence of alternative splicing, but the analogous set of neurons, is also pivotal for female reproductive behaviors (Demir & Dickson, 2005; Manoli et al., 2005; Stockinger, Kvitsiani, Rotkopf, Tirián, & Dickson, 2005). *fruitless* labeled neurons make up roughly 2% of the total nervous system. Very few morphological dimorphisms were found by the 2005 studies. However a more recent anatomical study by Cachero et al. (2010) used brain alignment to globally compare male and female *fruitless* neurons. As in this technique the brains of many specimen are warped onto one reference brain, it allows for a direct comparison of neural architecture; they found 19 new dimorphisms in various brain areas including the dorsal protocerebrum implied in female receptivity by the gynandromorph studies (Cachero, Ostrovsky, Yu, Dickson, & Jefferis, 2010; Yu, Kanai, Demir, Jefferis, & Dickson, 2010)

Efforts to understand the genetic determination of sex, and therefore the genetic basis of sexual dimorphisms, identified four crucial genes (reviewed in Baker, Taylor, and Hall 2001 and Manoli, Meissner, and Baker 2006). *Sex lethal (Sxl)* is only transcribed in the presence of two X chromosomes, i.e. only in genetic females. *Sxl* promotes the expression of the splicing factor *transformer (tra)*. The Tra-protein in turn will target the primary RNAs of the genes *doublesex (dsx)* and *fruitless* and promote alternate splicing into female forms. Absence of Tra-protein in males allows *dsx* and *fru* to be expressed into their male variants Dsx^M and Fru^M, whereas the presence of Tra-protein in the female will allow for alternate splicing of *dsx* into a Dsx^F-protein and a truncated *fruitless* pre-mRNA that is not translated to a

functional protein (Demir and Dickson 2005; reviewed in Baker, Taylor, and Hall 2001 and Manoli, Meissner, and Baker 2006).

Development of nervous systems that are able to appropriately mediate male and female courtship behaviors and the corresponding sex-specific morphology seems to primarily depend on the presence or absence, of these transcription factors (Fru and Dsx). Fru^M is only expressed in neurons and only in males. The repertoire of male behaviors largely depends on Fru^M, as males not expressing it, will not court, while females expressing it, will. (reviewed in (Anand et al., 2001; Demir & Dickson, 2005; Ito & Fujitani, 1996; Manoli et al., 2005; Yamamoto & Koganezawa, 2013). *dsx* seems to be of similar importance for the specification of the sex. However, it controls the development of both neuronal and other tissue in male and female, largely in concert with Fru, which is reflected in the large overlap of neurons expressing either transcription factor. Its expression is markedly dimorphic (Rideout, Dornan, Neville, Eadie, & Goodwin, 2010).

In the male, *fruitless* neurons are responsible for the majority of courtship related behaviors, (as well as for aggression (Vrontou, Nilsen, Demir, Kravitz, & Dickson, 2006)). They are, for example, in control of courtship song generation (von Philipsborn et al., 2011) and responsible for attraction to females by contact mediated chemosensation through receptors on the male forelegs (Thistle, Cameron, Ghorayshi, Dennison, & Scott, 2012). Yet Fru is not the only critical factor for the development of these circuits, as male *dsx* mutants court poorly and never sing or attempt copulation. In females *fruitless* neurons on the antennae innervate the Johnston's Organ (the fly ear) (Stockinger et al., 2005), probably sensing courtship song

(Vaughan, Zhou, Manoli, & Baker, 2014); and *fruitless* neurons in the uterus effect in great part, the drastic behavioral changes taking place after copulation (Häsemeyer, Yapici, Heberlein, & Dickson, 2009; Yang et al., 2009). These sex peptide sensory neurons (SPSN) are also *dsx* positive and silencing *dsx* neurons in the female renders her completely infertile (Rezával et al. 2012; Rideout et al. 2010). When inhibiting *dsx* neurons copulation occurs despite vigorous rejection and the sperm, although it reaches the spermatheca (specific receptacles) does not fertilize the egg, as no eggs are laid and no larvae hatch (Rideout et al., 2010).

But, although the *fruitless* and *doublesex* genes are the main players in specifying courtship-mediating circuits, there are others. In the male, the courtship song is affected by several mutations, *cacophony*, *dissonance* and *croaker* (reviewed in J. Hall 1994). A curious courtship influencing neuropeptide, which modifies male and female behavior, is SIFamide. It is expressed by only two (Terhzaz et al. 2007) to five (Carlsson et al. 2010) bilateral pairs of neurons, whose projections cover most of the brain, except the Mushroom Body (MB) complex. Lack of this peptide induced by RNAi or targeted cell ablation, results in extremely high receptivity with short latency in females and hyperactive males that vigorously court either sex (Terhzaz et al. 2007; Carlsson et al. 2010). Females mutant for painless (pain) exhibit a decrease in copulation latency (T Sakai, Kasuya, Kitamoto, & Aigaki, 2009). This gene codes transient receptor potential channels (TRP). Probably it is responsible for the control of rejection behaviors as mutants and knockdown specimen exhibit reduced levels of rejection, which therefore results in decreased copulation latency.

spinster, *icebox* and *apterous* are genes in which mutations lead to reduced female receptivity. The mutation *spinster*, was found to reduce receptivity and increase repelling behaviors (Suzuki, Juni, & Yamamotor, 1997). A more recent study identified two clusters involved in female mating decisions (Sakurai, Koganezawa, Yasunaga, Emoto, & Yamamoto, 2013). One cluster innervates the suboesophageal ganglion (SOG); the other is composed of second-order olfactory projection neurons, possibly relaying conspecific odor information to the lateral horn, via the mushroom body. The mutation *icebox* leads to reduced receptivity and an increase in rejection behaviors (Kerr & Ringo, 1997). More recent studies have shown that this is due to severe brain developmental defects, caused by a mutation that maps to the gene *neuroglian* which encodes an L1-type cell adhesion molecule (Carhan et al., 2005).

*apterous***,** from Greek for – without wings - is so called because a mutation in this gene leads to developmental defects, most notably malformed or absent wings. It is a LIM-homeobox transcription factor that is important for boundary formation during development. Flies mutant for *apterous*, are sterile, vitellogenesis is arrested (the uptake of yolk into the oocyte), and levels of female receptivity and Juvenile Hormone (JH) are reduced (J Ringo, Werczberger, Altaratz, & Segal, 1991; John Ringo, Werczberger, & Segap, 1992). JH, which is produced in the gland corpora allata (J Ringo et al., 1991), together with ecdysone orchestrates the maturation of a fly throughout its developmental stages. Vitellogenesis can be rescued by application of Juvenile Hormone (JH). Ringo et al. (1991) speculate that reduced receptivity is caused by reduced JH levels, which would evoke a delayed maturation. This in fact seems partly to be true as Bilen et al. (2013) found that lack of JH indeed slows down maturation and

thereby development of normal adult receptivity levels. Yet, in allatectomized females (genetic ablation of the corpora allata via diphtheria toxin), full maturation is reached already about 24 hours later than in controls (Bilen, Atallah, Azanchi, Levine, & Riddiford, 2013) and thus only explains reduced receptivity of very young females. Interestingly, allatectomy increased copulation latency by the factor five compared to controls and decreased the courtship index. This was shown to be due to a delay in pheromone production, namely the cuticular hydrocarbons 7, 11-C27 and 7,11-C29. (Bilen et al. 2013; reviewed in Riddiford 2012).

The Sensory Modalities of Courtship

Courtship consists of two parties each with a variable set of actions and possible sequence of their execution in mutual response to each other – it is extremely dynamic and complex. As representatives of their species both parties have the ultimate interest of producing a filial generation that is fit enough to achieve the same – courtship has developed to ensure this (Bastock & Manning, 1955; Dickson, 2008; J.-F. Ferveur, 2010).

A male will court almost anything, from a headless female to a moving, female-sized black dot (Agrawal, Safarik, & Dickinson, 2014). But given the option he will concentrate his efforts on a young but mature, conspecific virgin, one that is generally receptive to his advances and optimally fecund (Byrne & Rice, 2006; Long, Pischedda, Stewart, & Rice, 2009). He dispenses courtship willingly, but it does take its toll, especially the courtship song, and it will eventually render him less fit (Cordts & Partridge, 1996).

Conversely, the investment of the female into the next generation is much higher than that of the male. Therefore her interest in choosing a maximally fit conspecific must be more pronounced than the male's (Partridge & Fowler, 1990; T. Chapman, L. F. Liddle, J. M. Kalb, M. F. Wolfner, n.d.; Wigby, Chapman, Building, & Street, 2005). During the short time of courtship she must sample all reproductively relevant information that is presented to her.

What is known about the information that is exchanged during courtship? How is it exchanged? And which are the neurons that process it?

Vision

Vision is mostly important for the male in order to spot and pursue the female. In the dark or with blind males courtship latency is increased. Females in contrast do not change receptivity in light versus dark conditions (Takaomi Sakai, Isono, Tomaru, Fukatami, & Oguma, 2002). Given a choice, males prefer larger females, probably because the size of the abdomen correlates with more fecund ovaries (Long et al., 2009). The tradeoff is twofold: In a natural setting competition is already high and even higher for the very attractive large females. As a result fecundity of large females may be compromised due to continuous harassment by a contiguous sequence of courting suitors (Byrne & Rice, 2006; Long et al., 2009). Secondly, not only is the competition high, but also large females do require more courtship, i.e. for a longer time, until they reach acceptance (Turiegano, Monedero, Pita, Torroja, & Canal, 2012).

Audition

The ‘love song´ is possibly the most important cue for the female (Dickson, 2008). It informs her about fitness and especially the species of the male (Spieth, 1974). Receptivity of females courted by males with clipped wings is very low, playback of song rescues wild-type receptivity (Bennet-Clark & Ewing, 1967; Schilcher, 1976a, 1976b). Species and fitness appear to be encoded in a specific frequency pattern of the two elements of courtship song: The sine song and the pulse song. The sine song is a humming sound at a frequency of 140-170 Hz (Schilcher, 1976b). It is weakly enhancing receptivity; an effect is observable only after 15 minutes (C. P. Kyriacou & Hall, 1982). The pulse song’s carrier wave has a frequency of 150-300 Hz (Bennet-Clark & Ewing, 1967; Shorey, 1962), with pulses of amplitude modulation. The succession of pulses, interleaved with inter-pulse-intervals (IPIs), is characteristic for each Drosophilid species (C. P. Kyriacou & Hall, 1982). In *D. melanogaster* they each have a length of approximately 35 ms. Sine song and pulse song, each have a length of approximately 200 ms (C. P. Kyriacou & Hall, 1982). The intensity of pulse song episodes, i.e. its duration and frequency, is the aspect of song that increases receptivity (Talyn & Dowse, 2004). Furthermore, pulse song intensity seems to depend on female movement and vice versa: High levels of movement of her elicit pulse song from the male, while intense singing slows her down (Trott et al., 2012). Generally courtship song is rather invariable across *Drosophila melanogaster* populations (Gleason, 2005). Interestingly hybrid *melanogaster/simulans* females, prefer artificial song that is intermediate between the two species (C. Kyriacou & Hall, 1986).

Song is perceived by a part of the ~480 specialized neurons, forming an organ called Johnston's organ (JO), housed in the second antennal segment. Movement of the aristae and the third antennal segment are picked up by JO neurons - bipolar neurons that receive the movement as stretch - and then transmit it to the antennal mechanosensory and motor center (AMMC), a brain region that is located anterior-laterally, between antennal lobe (AL) and SOG (reviewed in Kamikouchi 2013).

Recently a study described substrate-borne vibratory signals, produced by males quivering with their abdomen. This behavior is only performed during courtship and coincides significantly with phases of female immobility. Males with clipped wings increase their quivering. However, this study did not measure female receptivity. Similar signals were also found in several other *Drosophila* species (Mazzoni, Anfora, & Virant-Doberlet, 2013).

Chemosensation

Olfactory information is sensed by olfactory receptor neurons (ORNs), expressing odorant receptors (ORs). They are housed in the 3rd antennal segment and the maxillary palp (reviewed in Vosshall and Stocker 2007). ORNs of about 50 different receptor classes project to the antennal lobe, where they terminate, in one of the ~50 glomeruli of the AL. Recently olfactory neurons were found that express a class of ionotropic receptors, called IRs (Benton, Vannice, Gomez-Diaz, & Vosshall, 2009). These neurons also project from the antennae to the antennal lobe. From here secondary projection neurons relay the olfactory information to the lateral horn, mostly via the MB ((Vosshall & Stocker, 2007). In the LH it arrives roughly segregated into fruity odors and potential pheromones (Jefferis et al., 2007).

Contact mediated chemosensation is processed by gustatory receptor neurons (GRNs), expressing gustatory receptors (GRs). They are mostly expressed on the proboscis, but also, somewhat surprisingly, on the rim of the wings, the legs and even in sensilla at the vaginal plates. Sensory neurons for gustation send their axons into the SOG (Vosshall & Stocker, 2007).

The cuticle of both sexes is covered with 30-50 types of cuticular hydrocarbons (CHC). Male and female share many of these, but each has their own CHC profile. One CHC, 7, 11-HD (7, 11 heptacosadiene), has been found to be of particular importance for female attractiveness: RNAi-mediated reduction of 7, 11-HD production increases courtship latency and reduces the courtship index; driving its expression in oenocytes of the male (pheromone producing cells) renders him attractive to other males. Furthermore 7, 11-HD is not produced by a sibling species *Drosophila simulans*, and thereby can serve as a crucial marker inhibiting heterospecific courtship towards *Drosophila* females (reviewed in Laturney and Billeter 2014).

The male foreleg tarsi have gustatory neurons housing the cognate receptor GR32a for three CHCs 7T (z-7-tricosene), 9T (z-9-tricosene) and 11T (z-11-tricosene) (Fan et al., 2013; Wang et al., 2011). Males have a 10-fold higher 7T concentration in their profile than females. This concentration difference helps male *D. melanogaster* distinguish between male and female (Fan et al. 2013). Moreover, other *Drosophila* species, also produce 7T or one of the similar tricosenes 9T or 11T, at concentrations which repel male *D. melanogaster* (Fan et al., 2013). Interestingly, GR32a neurons are neither *fruitless*-

positive nor do they make direct synaptic contact with *fruitless* neurons (Fan et al., 2013).

To the contrary, the ion channel coding genes *ppk23* and *ppk29* are also expressed in neurons on the foreleg tarsi, yet in *fruitless*-positive neurons. They have been implied as being crucial contact chemoreceptors for male-male repulsion and male-female attraction, yet their ligands are unknown. They are representatives of a class of ion channels called degenerin/epithelial sodium channel subunit family pickpocket thought to be involved in gustation and mechanoreception (Thistle et al., 2012).

The only known volatile pheromone to date is cis-vaccenyl acetate (cVA). It was initially described as an aggregation pheromone (Bartelt, Schaner, & Jackson, 1985), but is now shown to decrease courtship and promote aggression in males, and increase receptivity in females (Kurtovic, Widmer, & Dickson, 2007; Wang & Anderson, 2010). Its cognate receptor is OR67d (Ha & Smith, 2006). The respective, *fruitless-positive,* ORNs project to the DA1 glomerulus. This is one of the three sexually dimorphic glomeruli, DA1, VA1 and VL2a that are larger in males (Vosshall & Stocker, 2007). From here *fruitless*-positive projection neurons innervate the LH in a stereotypic, sex-specific manner and contact local *fru*+ third-order neurons of five different clusters, two of which have differing connectivity in male and female (Cachero et al., 2010; Datta et al., 2008; Kohl, Ostrovsky, Frechter, & Jefferis, 2013; Ruta et al., 2010).

While cVA increases receptivity through OR67d, expressed in *fru* neurons, a recent study showed that the intense and long-term exposure to cVA (several minutes to hours) activates OR65a, expressed in *fru*-negative neurons, which decreases female

receptivity for about a day (Lebreton et al., 2014). This appears to be the so called copulation effect, a sex peptide independent reduction of receptivity that is observed in the first 24 hours after copulation (A Manning, 1967).

Integration of Sensory Modalities

It is generally thought that receptivity results from the integration and accumulation of relevant information until a threshold is reached (Bastock & Manning, 1955). Central brain neuronal clusters were shown to regulate female receptivity by Zhou et al. (2014). These *dsx*-positive, and *fru*-negative neuronal clusters, pC1 and pCd, are cVA sensitive and courtship song sensitive (only pC1). When activated, receptivity is increased and when silenced receptivity is decreased.

The ventrolateral protocerebrum (VLP) that receives visual, gustatory and auditory information is discussed as a processing center for those modalities (Lai et al. 2012). An area that is formed by a dense network of *fru*-innervations, medial of the LH is hypothesized as multimodal processing center for tactile, auditory, gustatory, olfactory and visual information (Cachero et al. 2010). Information that is not directly courtship related, like the presence of food, egg-laying sites and other flies, is likely to play a role in courtship and may be processed in these centers

Internal Signaling Relating to Receptivity and Postmating Responses

During copulation seminal fluid is transported into the female's reproductive tract. The sperm is then stored in a specific organ, called

spermatheca, from which it will be released during the following days whenever an egg matured and arrived in the uterus (reviewed in Schnakenberg, Siegal, and Bloch Qazi 2012). Among many other seminal fluid proteins (SFP)(Avila, Sirot, LaFlamme, Rubinstein, & Wolfner, 2011) is sex peptide (SP), which is a 36 amino acids peptide, attached to the sperm cell (Chen, Stumm-Zollinger, & Aigaki, 1988; Peng et al., 1998). By binding to its cognate receptor (SPR), it induces several physiological and behavioral responses in the female (Yapici et al., 2008) called postmating responses (PMRs). Most importantly two behaviors are altered: Receptivity is extremely reduced and egg-laying is extremely increased. These postmating responses revert to the pre-copulatory state when the sperm is used up and there is no more SP left in the spermatheca (Aubrey Manning, 1962; Peng et al., 1998).

Some studies indicate that SP is not the only SFP that is important in mediating the full postmating response (Ram & Wolfner, 2007; Smith, Sirot, Wolfner, Hosken, & Wedell, 2012). The SFP ovulin for example is necessary for the full postmating response of ovulation stimulation. Females mated with ovulin knock-down males exhibit significantly reduced egg laying during the first 24 hours after mating (Herndon & Wolfnert, 1995; Ram & Wolfner, 2007).

In two by now seminal studies, published back-to-back in 2009, Häsemeyer et al. and Yan et al. demonstrated sufficiency of only a few neurons in the reproductive tract to sense SP, for a majority of the postmating responses to be switched on (Häsemeyer et al., 2009; Yang et al., 2009). Both studies found four bilateral *fru*- and *ppk*-positive neurons, three on each side of the uterus wall, near the spermatheca, and one more per lateral oviduct. Later it was shown

that the six uterus neurons also intersect with *dsx* and that they are sufficient for PMRs as well as probably cholinergic (Rezával et al. 2012). The processes of these neurons innervate the ventral nerve cord (VNC). Silencing of these neurons with the chemical synapse inhibitor shi^{TS} (driven by *ppk-GAL4*), resulted in postmating responses – reduced receptivity and increased egg-laying. Yang et al. (2009) furthermore reported that the G-protein-coupled sex peptide receptor (Yapici et al., 2008) is likely to silence these neurons via the inhibitory G-protein Gαi, which reduces cAMP levels (by inhibiting adenylate cyclase) and possibly via cAMPs downstream effector PKA (protein kinase A).

In an effort to identify neurons that process PMRs in the abdominal ganglion (abg), Rezával et al. (2012) screened a collection of enhancer-trap FLP lines and found one line, $ET250^{FLP}$, which intersected with *dsx-GAL4*. Activation of the cells of this intersection with *UAS-TrpA1* resulted in postmating responses. Anatomical analysis revealed, that of the 27 cell bodies located in the abg, four send axons into the SOG and two send axons into the uterus, while the remaining ones resemble interneurons (Rezával et al. 2012). Furthermore in a 2014 study they manipulated the intersection between *dsx-FLP* and *Tdc2-GAL4* (which labels octopaminergic neurons). Upon activation they observed postmating responses in virgins; upon silencing they observed the inhibition of PMRs in mated females. These effects are analogous to the ones observed with the *dsx-GAL4* ∩ $ET250^{FLP}$ intersection and thus consistent with the suggestion that they are (at least partly) the same neurons, as well as octopaminergic (Rezával, Nojima, Neville, Lin, & Goodwin, 2014).

The specific cluster of second order SP sensory neurons was found by Feng et al. (2014). By screening the VT collection of enhancer GAL4-lines and subsequent stochastic labeling, they described a cluster of cell bodies in the abg, that they termed SAG (SP abg) with dendrites connecting to SPSN and with axons projecting to the brain and arborizing perioesophageally and in the dorsal protocerebrum. Silencing SAG neurons, which are *dsx*- but not *fru*-positive, induces postmating behaviors.

A set of neurons relevant for female behavior during courtship that does not relate to the postmating responses has been uncovered by Bussel et al. (2014) This set is defined by the promoter for a homeobox transcription factor called *Abdominal-B (Abd-B)* (Bussell et al., 2014). RNAi-mediated knockdown of Abd-B protein reduces receptivity. Rejection behaviors (ovipositor extrusion) and egg-laying are not increased, which implies a mechanism of receptivity reduction, different from the postmating switch. The cell bodies (~142) are located in the abg (and only few in the reproductive system), from where they send ascending neurites to the brain (SOG, ventrolateral and superior neuropils) and reproductive organs. They control aspects of female locomotion during courtship, i.e. pausing. Females with *Kir2.1*-silenced *Abd-B* neurons paused significantly less than controls. This behavior seems to depend on multiple cues, but prominently so on courtship song.

Aims: The Uncovering of Neural Sets in Receptivity and their Functional and Anatomical Characterization

The elucidation of the functional architecture of neural circuits and its logic is of utmost interest in the pursuit of understanding how

behaviors are mediated by their neural substrate. To this end courtship is an ideal behavioral paradigm. We focus on receptivity and aim to find neural sets mediating it (Chapter Two) and to functionally and anatomically characterize these sets (Chapter Three).

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II. *apterous* Neurons Involved in Female Receptivity

Summary

Drosophila courtship is an established paradigm to study basic principles of how neuronal circuits produce behavior. We wanted to investigate neurons that are involved in a virgin female's decision whether to accept a courting male for copulation or not, her receptivity. Employing a single-pair receptivity assay and a temperature-inducible neuronal inhibitor, we screened eleven GAL4-lines for the effect of silenced neurons on female receptivity. We found that silencing *apterous-GAL4* neurons strongly reduces receptivity. We show that this effect cannot be explained by blindness, diminished attractiveness, compromised locomotion or the absence of Apterous in the adult. Silencing apterous neurons results in two further phenotypes: Firstly, reduced egg-laying, which indicates that silencing *apterous-GAL4* neurons does not trigger the postmating switch, and secondly, a constitutively extended proboscis and bloatedness, which we show does not explain reduced receptivity. We analyzed the expression pattern of *apterous-GAL4* and found that the antennal lobe (first relay of olfactory information), the antennal mechanosensory and motor center (first relay of auditory information) and sex peptide sensory neurons are not labeled.

Introduction

For the elucidation of basic neural mechanisms of simple systems, innate behaviors are a useful subject. They are mediated by genetically encoded, hard-wired circuits and thus every tested individual behaves through the action of nearly identical circuits. The fact that genetic programs construct the circuitry of neural networks, which in turn coordinate behavior, allows for the understanding of the interplay between these three levels of organization. In addition, a versatile array of tools has been developed during the last decade to investigate neural circuits and behavior in *Drosophila melanogaster* (reviewed in Kazama 2014).

Courtship behavior provides an excellent paradigm for the investigation of how behavior is generated. A series of behaviors is performed by the male, to which the female responds and if she is receptive, it will culminate in copulation. If the female is in the male's visual field he will orient himself towards her. This is a baseline behavior, whatever courtship action he is performing, he will not let her out of his sight (Bastock & Manning, 1955). Then he will touch her with his forelegs, sing with vibrations of his wing and quivering of his abdomen (Mazzoni, Anfora, & Virant-Doberlet, 2013; Spieth, 1974). Eventually he will contact her abdomen with his proboscis (licking) and attempt copulation. This procedure is a highly dynamic interaction between male and female. The female decamps and stops, then runs away again, occasionally fending with her hindlegs and extruding her ovipositor in rejection. The male tends to run after her and, in phases of pausing, to circle her singing (reviewed in J. Hall 1994).

During courtship, the female is exposed to several sensory cues, based on which she will make her decision (Dickson, 2008). If she is a mature virgin courted by a conspecific, her receptivity will be high – if she is recently mated or is being courted by a male of a sibling species, it will be low (Spieth, 1974). Upon mating, females display a set of behavioral changes termed postmating responses (PMRs), which are characterized by drastically reduced receptivity and increased egg laying (Bastock & Manning, 1955; Yapici, Kim, Ribeiro, & Dickson, 2008).

Valuable insights have been gained by recent investigation of the neural circuits that control the male courtship behavior (McBride et al., 1999; Redt-Clouet et al., 2012; Stockinger, Kvitsiani, Rotkopf, Tirián, & Dickson, 2005; Toda, Zhao, & Dickson, 2012; von Philipsborn et al., 2011), whereas the female behavior was, until recently underrepresented. We asked a simple question: Which neurons are involved in female receptivity? We addressed this question by silencing different sets of neurons of the female during courtship between naïve couples and measure receptivity.

In an initial screen we found that silencing *apterous* neurons reduces the average receptivity by ~50% compared to control levels. Apterous is a zinc-finger transcription factor that is active throughout development. Apterous mutants have been shown to exhibit reduced receptivity but the role of *apterous* neurons in this phenotype has not been clear.

In this work we show that activity in *apterous* neurons is required for normal levels of receptivity. To control for intact gross locomotion we tested if females move at normal levels and found that they do. Moreover we wanted to know whether females are attractive to males

and elicit levels of courtship, comparable to those elicited by controls. Our results show no difference in courtship levels. Examination of the *apterous-GAL4* expression pattern revealed that neither brain areas responsible for auditory processing, nor areas for olfactory processing are labeled. To control for the effects of impaired vision by *apterous* neuron silencing, we tested the receptivity of flies with silenced *GMR-GAL4* neurons and found that receptivity is not affected. Furthermore we investigated egg-laying of *apterous* silenced females in order to test whether by silencing *apterous* neurons we target neurons involved in effecting PMRs. Our results suggest that *apterous-GAL4* neurons are not involved in the PMRs. Knock-down of Apterous protein in the adult indicates that presence of Apterous is not required for wild-type receptivity behavior.

This work lays the ground for further investigation of *apterous* neurons. It will potentially help to identify novel neurons that play a role in mediating receptivity and to understand the mechanism by which they do so.

Results

The Screen

We tested the effects of neuronal silencing on female receptivity of eleven GAL4 lines. Silencing was achieved by the expression of the inwardly rectifying potassium channel 2.1 (Kir2.1) in the respective cells, under the control of a ubiquitously expressed (via a tubulin promoter), temperature-sensitive GAL80 (*TubGAL80^{TS}*). This construct allows flies to develop without any neuronal manipulation when kept at 18˚C – GAL80 binds GAL4 and prevents it from binding to UAS (upstream activating sequence). Upon exposure to 30˚C, the

GAL80^{TS} becomes instable; GAL4 can bind to UAS and drive expression of Kir2.1. The results of this screen are shown in Figure 2.1. The GAL4 lines were chosen for potentially being involved in receptivity with the exception of one line that was created by us, the enhancer-trap line *DH1-GAL4* (see Material and Methods). Given the importance of olfaction in mating behavior, several of these lines label cells of the olfactory system. OR83b is a co-receptor of most olfactory receptor channels; it labels 70-80% of the sensory neurons projecting from the antennae to the antennal lobes (AL) (Larsson et al., 2004). *GH146* labels roughly 60% of the projection neurons (PN), projecting from the antennal lobe to the lateral horn (LH). These projections include most PN classes (Potter & Luo, 2010). OR67d is a receptor that binds the volatile pheromone 11-*cis*-Vaccenyl acetate (cVA) which is produced by the male and promotes male-male repulsion and female-male attraction. We tested *OR67d-GAL4* as well as an OR67d mutant (Ha & Smith, 2006; Kurtovic, Widmer, & Dickson, 2007). *NP6099-GAL4* labels two to three cells in the LH (as well as the lamina of the optic lobe) (Tanaka, Awasaki, Shimada, & Ito, 2004; Umetsu, Murakami, Sato, & Tabata, 2006). *MZ19-GAL4* labels PNs of DA1, VA1d and DC3 (Ito, Suzuki, & Estes, 1998; Jefferis et al., 2004) and *NP5194-GAL4* (Hayashi et al., 2002) also labels cells in the LH. *Poxn-GAL4* expresses GAL4 in gustatory bristles (Krstic, Boll, & Noll, 2009). The line *NPF-GAL4*, labels cells that express neuropeptide F, a *Drosophila* homolog to vertebrate neuropeptide Y; it has a sexually dimorphic expression pattern (G. Lee, Bahn, & Park, 2006). Lastly, *apterous-GAL4* was chosen because *apterous*-mutant females exhibit reduced levels of receptivity (Ringo, Werczberger, Altaratz, & Segal, 1991).

Figure 2.1 shows the cumulative percentages of receptivity of these lines. Black bars represent the receptivity of control flies that were not exposed to 30˚C and thus expression of Kir2.1 was not induced. Grey bars represent the receptivity of flies that were exposed to 30˚C and consequently have the respective neurons silenced by the action of Kir2.1. No statistically significant difference between control and experimental conditions could be observed in any of the lines, with the exception of *OR83b-GAL4* (p=0.0339) and *apterous-GAL4* (p<0.0001).

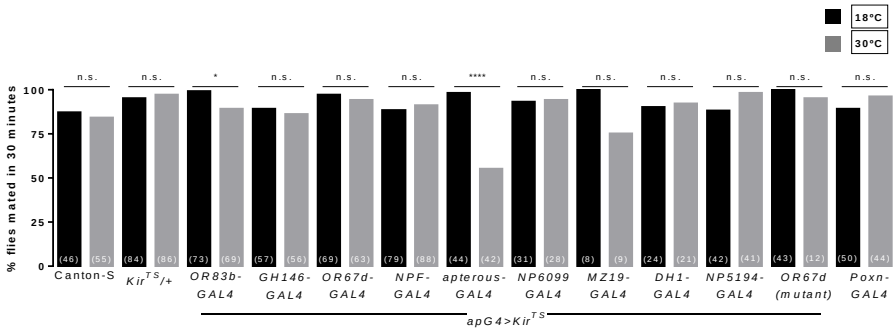

Figure 2.1 Receptivity of lines tested in the screen; **silenced *apterous-GAL4* neurons exhibit reduced receptivity.** Receptivity of lines tested in the screen, plotted as cumulative percentage of copulation events across couples during 30 minutes. Control condition is in black, experimental condition in grey. *p<0.05, **p<0.01 ***p<0.001***p<0.0001, n.s.=not significant, Fisher's exact test; sample size in in brackets. *apG4=apterous-GAL4*; *Kir^{TS}=UASKir2.1, TubGAL80^{TS}*

In order to confirm the results obtained in the pilot screen, we retested *apterous-GAL4* and the previously observed reduction of receptivity ~50% persisted (p<0.0001; Figure 2.2). We decided to continue examining the line with the stronger phenotype, *apterous-GAL4*.

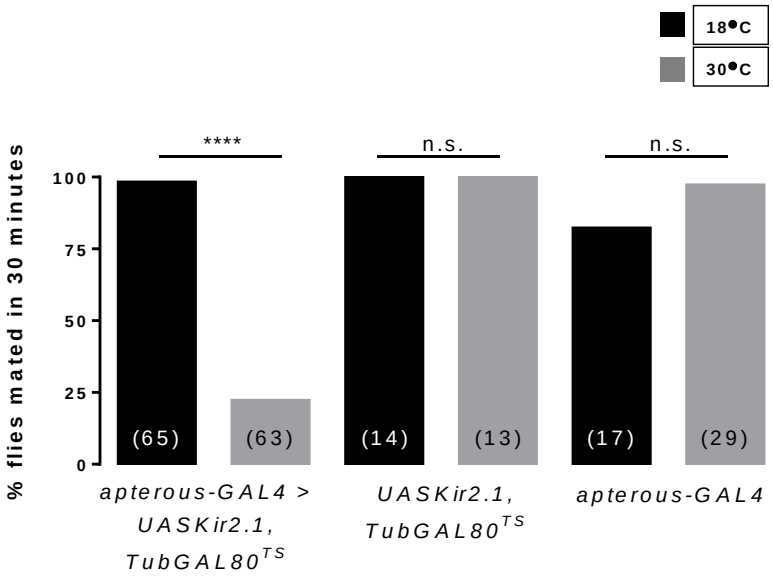

Figure 2.2. *apterous-GAL4* phenotype persists after repeating the experiment Receptivity of *apterous-GAL4 > UASKir2.1, TubGAL80^{TS}* and respective controls, plotted as cumulative percentage of copulation events across couples during 30 minutes. Control condition is in black, experimental condition in grey. Presented data was acquired in experiments independent from the data in Figure 2.1. ****p>0.0001, Fisher's exact test. Sample size in brackets.

Furthermore we were interested in the effects a constitutive expression of different neural silencers may have when driven by *apterous-GAL4.* Therefore we tested *apterous-GAL4* or *nsyb-GAL4* driving expression of *UASKir2.1* and *apterous-GAL4* or *nsyb-GAL4* driving expression of *UAS-TNT* (an inhibitor of synaptic transmission; (Sweeney, Broadie, Keane, Niemann, & O'Kane, 1995)). Note that we did not use the temperature sensitive GAL80 here. In all four cases flies of the respective genotypes were not viable, possibly the silencing of too many neurons, from too early on did not allow for the development of the eggs into viable stages of the organism.

The Expression Pattern

In order to examine the expression pattern of *apterous-GAL4*, we expressed a membrane bound GFP (*UAS-mCD8-GFP*) under its control (Figure 2.3). We do not observe staining of the antennal lobe (AL) – the first relay of olfactory information (Vosshall & Stocker, 2007) – nor of the antennal mechanosensory and motor center (AMMC), an area that receives information about air movement, including sound (Kamikouchi, 2013)(Figure 2.3A). This implies that *apterous-GAL4* silencing does not interfere with the perception of courtship song or sex pheromones. However, many neurons are labeled, in fact a few hundred, not including the optic lobes and the mushroom body. Moreover, the optic lobes and to a lesser degree the mushroom body are strongly labeled. Areas labeled by innervations with fine processes are discernible throughout the brain and VNC (Figure 2A and 2B). A detailed description of the *apterous-GAL4* expression pattern will be presented in Chapter III. Outside the brain and VNC we can recognize two to four neurons in dorsal sensilla at the tip of the abdomen (Figure 2C). Their neuronal processes project in proximity of the reproductive organs and innervate the tip of the abdominal ganglion. The anatomy of the sex peptide sensory neurons whose cell bodies are located at the uterus and oviduct (Häsemeyer, Yapici, Heberlein, & Dickson, 2009; Yang et al., 2009) however, is different from these abdominal *apterous* neurons, indicating that they are novel.

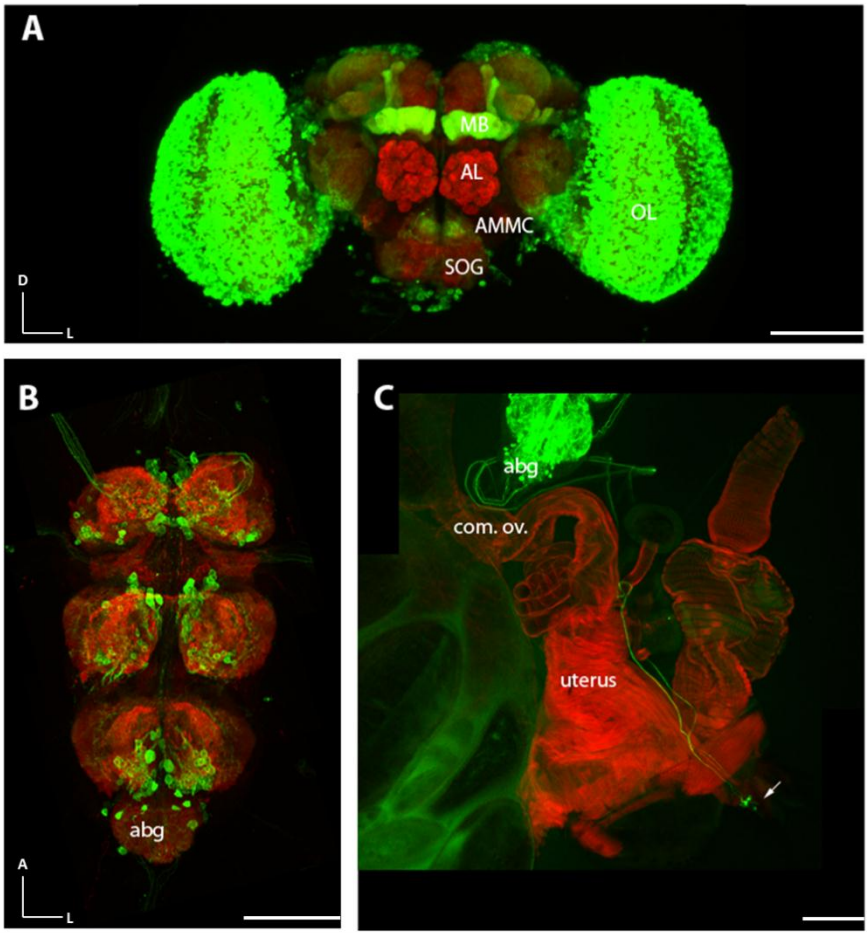

Figure 2.3. Expression pattern of *apterous-GAL4* in brain, ventral nerve cord (VNC) and reproductive system
(A, B, C) Representative images of the *apterous-GAL4* expression pattern; *apterous-GAL4>UAS-mCD8-GFP* staining in green, neuropil staining with NC82 in red **(A, B)**. Image of the brain was taken at 20x **(A)**; three images taken at 63x were stitched together to show a whole VNC (Fiji plugin) **(B)**. 10x image of the reproductive tract and adjacent areas **(C)**. Phalloidin labeling muscle tissue in red. *apterous-GAL4>UAS-mCD8-GFP* in green. Arrow points to two to four peripheral *apterous* neurons. common oviduct (com. ov.). Scale bars, 100µm

The Phenotype is Neuronal

We wanted to exclude the possibility that expression of the inwardly-rectifying potassium channel Kir2.1 (Baines & Uhler, 2001) in cells

other than neurons has effects on physiology that may change the behavior of the animal. To test this we used *elav-GAL80* (Rideout, Dornan, Neville, Eadie, & Goodwin, 2010) together with *apterous-GAL4* and *UASKir2.1, TubGAL80^{TS}*: *Elav-Gal80* blocks *apterous-GAL4* driven expression of Kir2.1 in all neurons. If receptivity reduction is purely neuronal, we will observe a rescue of receptivity to wild-type levels. In fact, we did observe exactly this: no significant difference between control and experimental condition (p=1.000); wild-type behavior was fully rescued (Figure 2.4). This is strong evidence that the phenotype is caused by inhibition of neurons.

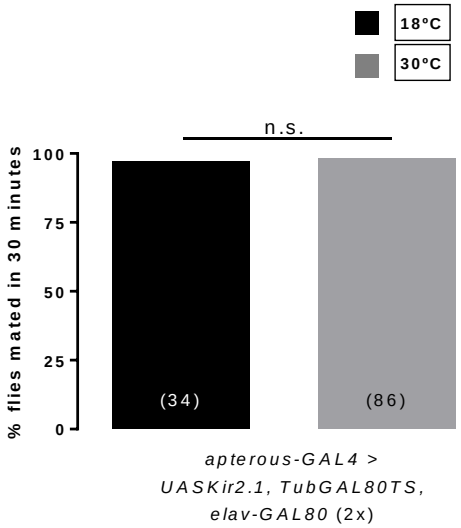

Figure 2.4. Rescue of receptivity to control levels with *elav-GAL80*: apterous phenotype is neuronal
Receptivity of *apterous-GAL4 > UASKir2.1, TubGAL80^{TS}, elav-GAL80* (2 copies) plotted as cumulative percentage of copulation events across couples during 30 minutes. Control condition is in black, experimental condition in grey. n.s. p=1.000, Fisher's exact test, n(18)=34 and n(30)=86; sample size in brackets.

The Locomotion

In another control experiment we wanted to test the possibility that silencing neurons leads to unspecific effects on behavior, effects not directly related to receptivity. For this we decided to test gross locomotion. We quantified the total number of millimeters walked by a single virgin female within the arena during 1 minute (Figure 2.5). We found no significant difference between experimental and control conditions (p=0.2331). We conclude that gross locomotion is not influenced by silencing *apterous* neurons. However, we cannot rule out the possibility that subtle locomotion behaviors are affected.

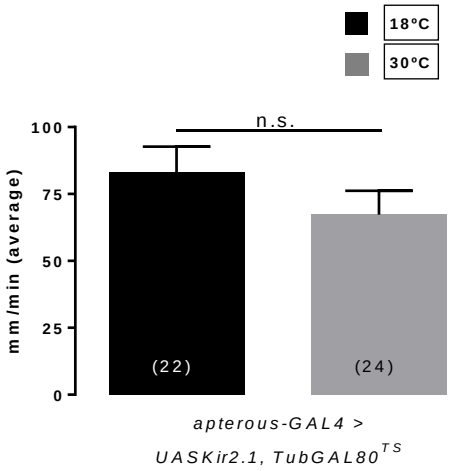

Figure 2.5 Gross locomotion is not influenced by silencing *apterous* neurons
The locomotion ability of female flies with silenced apterous neurons is not significantly compromised, as measured by the total distance walked in the arena during 1 minute using custom developed software. Error bars denote SEM. n.s. p=0,2331, student t-test; sample size in brackets.

The Courtship Index

In order to test whether females with silenced *apterous* neurons are attractive to males and able to elicit levels of courtship comparable to controls, we measured the courtship index. The courtship index is the

fraction of time spent by males performing courtship behaviors within 10 minutes or until copulation.

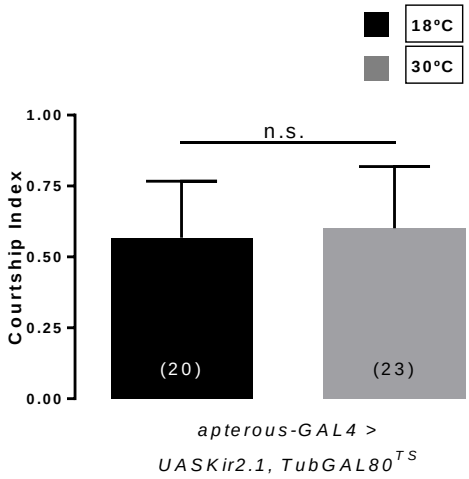

Figure 2.6 Female capability of triggering male courtship behavior is unchanged by silencing *apterous* neurons
Courtship index serves as a measure for the attractiveness of females. It is the fraction of time spent by the male with courtship actions during the first 10 minutes or until copulation. Every courtship related action, i.e. orienting, following, singing, was counted using courtship videos and a stopwatch. Error bars denote SEM. n.s. p=0,6071, student t-test; sample size in in brackets.

We did not observe a significant difference in the courtship index of males courting control females compared to experimental females (p=0.6071; Figure 2.6). We conclude that silencing *apterous* neurons in females does not change their attractiveness towards males.

Visual Contribution to Receptivity

apterous-GAL4 strongly labels the optic lobes so we wanted to test the possibility that reduced receptivity is caused by females being blind. Vision is not thought to be important for female receptivity, yet we wanted to confirm this notion by using *GMR-GAL4* in our setup, a line that labels photoreceptor neurons (Hay, Wolff, & Rubin, 1994). Silencing those cells, using *UASKir2.1, TubGAL80^{TS}* should render

the females blind. Our results suggest that vision is not required for female receptivity and that blindness is not causing the reduced receptivity of flies with silenced *apterous* neurons (p=0.3764; Figure 2.7; Kim, Jan, and Jan 2012).

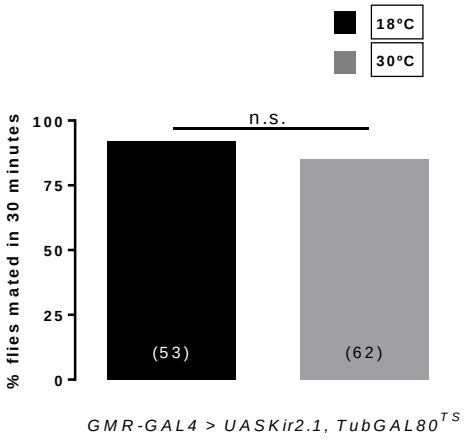

Figure 2.7. Silencing photoreceptor neurons via *GMR-GAL4*-driven expression of Kir2.1 does not change female receptivity.
Receptivity of *GMR-GAL4 > UASKir2.1, TubGAL80^{TS}* flies, plotted as cumulative percentage of copulation events across couples during 30 minutes. Control condition is in black, experimental condition in grey n(18˚C)=53 and n(30˚C)=62, n.s. p=0,3764, Fisher's exact test; sample size in brackets.

Presence of Apterous

Apterous activity as a LIM-homeobox transcription factor is important during development, where it plays a role in boundary formation, is essential for wing development and confers neuronal identity. Adult *apterous* mutants exhibit a reduced receptivity defect (Benveniste, Thor, Thomas, & Taghert, 1998; Herrero, Magariños, Torroja, & Canal, 2003; Herzig, Thor, Thomas, Reichert, & Hirth, 2001; Ringo et al., 1991). We wondered whether Apterous activity is required for normal behavior in the adult. We tested this by knocking down expression of the Apterous protein only in the adult.

We created flies carrying the following transgenes: *UASdcr2*; *apterous-GAL4*, *apterous-GFP*, *apterous-RNAi* and *TubGAL80^{TS}*. These flies will express *apterous-RNAi* in *apterous-GAL4* cells when exposed to 30˚C (controlled by *TubGAL80^{TS}*). Apterous will be knocked-down, as well as GFP tagged Apterous (apterous-GFP) allowing for an estimate of knockdown efficacy. The transgene *UASdcr2* has been shown to increase knockdown efficacy (Baumgardt, Miguel-Aliaga, Karlsson, Ekman, & Thor, 2007). The *apterous-RNAi* worked, as flies grown from eggs kept at 30˚C developed no wings, confirming that *apterous-RNAi* knocked-down the protein during development to critical levels. Checking for Apterous-GFP fluorescence allowed us to estimate the degree to which the knockdown of GFP-tagged Apterous was effective after different times of exposure to 30˚C. We exposed flies to 30˚C for times ranging from 24 hours up to 10 days in one day steps. We observed small fluctuations in all samples, at a very low level of fluorescence, usually only affecting the optic lobes, with a tendency to be lower after longer exposures. In order to choose a compromise between maximal knockdown and maximal health of the flies we decided to expose flies to 30˚C for 96 hours (see Appendix A). When tested for receptivity adult females, after four days of RNAi-expression, exhibited no statistically significant difference to control groups suggesting that Apterous is not necessary for wild-type receptivity in adult flies (Figure 2.8).

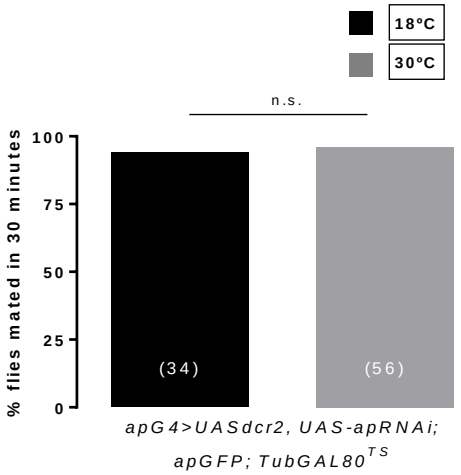

Figure 1.8 Knockdown of Apterous in the adult fly does not alter receptivity
Flies that express *dcr2* and *apterous-RNAi* (when exposed to 30˚C for 96 hours) do not show reduced receptivity; plotted as cumulative percentage of copulation events across couples during 30 minutes. Control condition is in black, experimental condition in grey; n(18˚C)=34 and n(30˚C)=56, n.s. p=0,6312, Fisher's exact test; sample size in in brackets. *ap=apterous*

Egg-Laying

Silencing *apterous* neurons reduces female receptivity. Is it possible that by silencing *apterous* neurons we triggered the postmating switch? To test this we examined egg-laying. If it is true that we induce postmating responses by silencing *apterous* neurons, we expect to observe an increase in egg-laying in the virgin female.

Indeed we observe an effect on egg-laying, but in the opposite direction as the one expected if the postmating switch was induced (Figure 2.9). Our results show a weak but still significant reduction of egg-laying in virgins ($p<0.05$). Furthermore, in mated flies we see a significant reduction of egg-laying, in fact by approximately 70% ($p<0.0001$). This implies a mechanism of receptivity reduction that is different from the postmating switch.

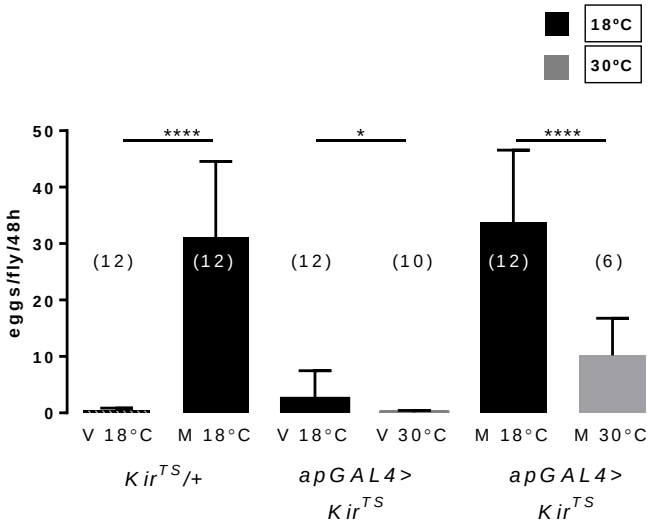

Figure 2.9 Silencing *apterous* neurons reduces egg-laying
Egg-laying of *apterous-GAL4 > UASKir2.1, TubGAL80^{TS}*, plotted as the average of eggs laid per fly per 48 hours. Control condition is in black, experimental condition in grey. Egg laying of virgins with silenced apterous neurons is slightly reduced or unchanged. Egg-laying of mated flies with silenced *apterous* neurons is strongly reduced. Sample sizes are indicated in brackets on the respective column (n of 1 equals one plate with 5 flies). *p<0.05, **p<0.01 ***p<0.001***p<0.0001, n.s.=not significant, student t-test. *apGAL4= apterous-GAL4; Kir^{TS}=UASKir2.1, TubGAL80^{TS},* V=virgin, M=mated

Proboscis Extension and Bloated Abdomen

A third, morphological, phenotype resulting from silencing *apterous-GAL4* neurons was a bloated abdomen and a continuously extended proboscis in a majority of the flies (see Appendix B). Flies with a bloated abdomen always had an extended proboscis but the opposite was not always true. In order to ascertain that this combined phenotype is not causing reduced receptivity, we split experimental flies into two groups: one with extended proboscis and the other without extended proboscis (Figure 2.10). Then we tested the statistical significance of the difference of each group compared to control flies. The difference diminished, but stayed extremely

significant (p=0.0001). We can conclude that these phenotypes do not explain reduced receptivity. However, we cannot exclude a slight contribution of this phenotype to the receptivity phenotype.

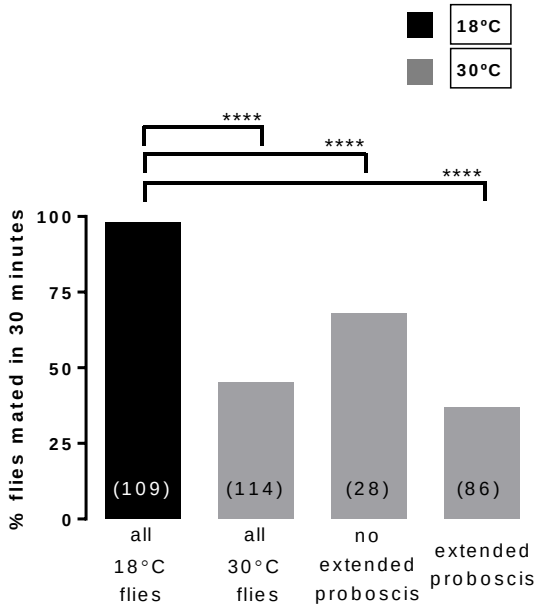

Figure 2.10 Flies of wild-type morphology also show reduced receptivity
Receptivity of *apterous-GAL4 > UASKir2.1, TubGAL80^{TS}* plotted as cumulative percentage of copulation events across couples during 30 minutes. Experimental flies are split into two groups, one containing flies without extended proboscis, the other containing flies with extended proboscis. Videos of the receptivity screen were used. Control condition is in black, experimental condition in grey. Sample sizes are indicated in brackets on the respective column. *p<0.05, **p<0.01 ***p<0.001***p<0.0001, n.s.=not significant, Fisher's exact test.

Discussion

In this chapter we have presented data indicating that *apterous* neurons play a crucial role in the mating decision of female *Drosophila* in response to male courtship display.

In the course of this work we have demonstrated that reduced receptivity results from silenced *apterous* neurons, not non-neuronal cells, and that silencing of the optic lobe does not contribute to this phenotype. Furthermore, we examined gross locomotion and saw that it is not influenced by silenced *apterous* neurons. Moreover, the attractiveness of the female towards the male is not reduced. The presence of Apterous protein in the adult is not required for receptivity, as RNAi-mediated knockdown of Apterous has no effect in the adult female. Furthermore, silencing of *apterous* neurons in virgin flies does not increase egg-laying. To the contrary, egg-laying is reduced in virgin and in mated females. This suggests that neurons outside of the described SP-circuitry are involved in evoking this phenotype. We note that flies with silenced *apterous* neurons have a grossly bloated abdomen and a continuously extended proboscis, which however does not by itself explain the receptivity defect.

The results of this work have shown that the reduced receptivity observed by Ringo et al. (1991), is not exclusive to *apterous* mutants. Neural function of *apterous-GAL4* labeled neurons is required for wild-type receptivity levels. This offers a new tool – *apterous-GAL4* – at our disposal in the task of understanding courtship in *Drosophila*.

Further studies will have to reduce the number of neurons that are silenced, in order to identify the neurons that are controlling this type of receptivity.

Materials and Methods

Fly Stocks

Flies were reared on a standard medium at 18ºC in a 12 hour light / 12 hour dark cycle.

We created the enhancer-trap line *DH1-GAL4*, by transposon hopping using the transposase Δ2-3 and transposon acj6 (Bourbon et al., 2001)

Stock List:

acj6 (Komiyama, Johnson, Luo, & Jefferis, 2003)

apterous-GAL4 (Stevens & Bryant, 1985)

apterous-GFP (Müller et al., 2010)

Δ2-3 (Laski, Rio, & Rubin, 1986)

GH146-GAL4 (Wilson, Turner, & Laurent, 2004)

GMR-GAL4 (Hay et al., 1994)

MZ19 (Ito, Suzuki, and Estes 1998)

NP5194 (Hayashi et al., 2002)

NP6099 (Tanaka et al., 2004)

NPF-GAL4 (G. Lee et al., 2006)

OR67d (mutant) (Fishilevich & Vosshall, 2005)

OR67d-GAL4 (Fishilevich & Vosshall, 2005)

OR83b-GAL4 (Larsson et al., 2004)

Poxn-GAL4 (Krstic et al., 2009)

TubGAL80^{TS} (McGuire, Le, Osborn, Matsumoto, & Davis, 2003)

UAS-ap-RNAi (Vienna Drosophila Resource Center)

UAS-dcr2 (Baumgardt et al. 2007)

UAS-Kir2.1 (Baines & Uhler, 2001)

UAS-mCD8-GFP (T. Lee & Luo, 1999)

Neural Manipulation and Receptivity Assay

We used a temperature-inducible genetic system to silence specific sets of neurons in the adult female and then tested the effect of neural silencing on female receptivity (Baines & Uhler, 2001). Neuronal manipulation was achieved by the expression of Kir2.1, an inwardly rectifying potassium channel whose expression causes hyperpolarization of the neurons (Baines & Uhler, 2001). Kir2.1 expression was targeted to subsets of cells using the GAL4/UAS system (Brand & Perrimon, 1993), which utilizes the yeast derived transcription activator protein GAL4 and its target sequence, the "upstream activating sequence" (UAS). Insertion of GAL4 into the Drosophila genome (possible by different means) in front of a driver leads to the expression of GAL4 in a defined set of cells. If an engineered sequence is present in the genome that contains the UAS and a transgene of choice, GAL4 can bind to UAS and drive expression of the transgene that follows the UAS. A TubGAL80^{TS} transgene was added in order to gain temporal control over Kir2.1 expression. At low temperature (18ºC), GAL80^{TS} will be expressed and will prevent GAL4 binding to the UAS sequence, whereas at high temperature (30ºC) GAL80^{TS} will be inactive allowing the expression of any effector gene driven by GAL4. This technique is also known as TARGET (temporal and regional gene expression targeting) (McGuire et al., 2003).

Flies were reared on a standard medium at 18ºC in a 12 hour light / 12 hour dark cycle. Virgin females and virgin males were collected at eclosion under CO_2 anesthesia and then kept singly in vials at 18˚C. In experimental conditions silencing was induced by incubation of flies for ~15 hours at 30˚C, followed by an additional incubation of ~24 hours at 25˚C for acclimatization. Flies under control conditions were always kept at 18˚C and were then joined with the experimental flies at 25˚C 24 hours prior the behavioral experiment. A schematic representation of the different temperature treatments can be visualized in Figure 2.11. Experiments were subsequently performed between 0900h and 1300h at 25˚C and 60% humidity with females aged for 8-16 days and males aged for 4-8 days (their developmental age was less because of several days spent at 18˚C). Male and female flies were coupled into arenas (16mm x 4mm, diameter x depth) using an aspirator and then filmed for 30 minutes using a commercial color video camera (Sony Models: HDR-XR520VE, HDR-CX570E, HDR-SR10E).

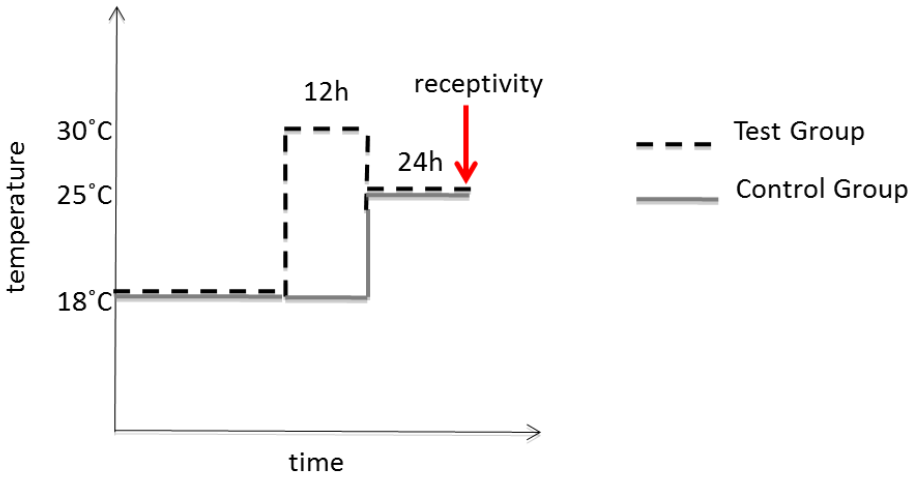

Figure 2.11 Schema of the temperature regime for Kir2.1 expression for receptivity experiments

Maximally 24 arenas were recorded in one film. A light plate was used as light source. The movies were viewed using Sony software ("PlayMemories Home"). Time of arena-placement and time of copulation were scored manually and analyzed with Microsoft Excel 2010 and GraphPad Prism 6.

Wild-type flies of the Canton-S (CS) strain were used to control for the effect of the different temperature treatments on receptivity behavior. No alterations in the mating levels were seen in CS flies that were subjected to the experimental conditions (30ºC) as compared with control conditions. Flies carrying *fru^{M}-GAL4* and *UASKir2.1, TubGAL80^{TS}* transgenes were used to control for the efficiency of neuronal silencing. In experimental conditions they exhibited a reduction to ~5% receptivity. This evidences that our experimental design works.

Staining Protocol

Flies were dissected in PBS (phosphate buffered saline) on a sylgard coated dishes and the brains stored in PBS on ice for up to an hour, using 0.5 mL Eppendorf tubes. Dissected brains and VNCs were fixed by incubation with a 4% paraformaldehyde solution (in PBS) for 30 minutes at RT. The PFA was removed and the fixed tissue incubated in 10% normal goat serum (NGS; in PBS) for 15 minutes and was followed by incubation with antibodies. Antibodies used included: rabbit anti-GFP 1:2000 (v/v) (Invitrogen); mouse anti-bruchpilot (NC82), 1:10 (v/v) (Developmental Studies Hybridoma Bank at the University of Iowa); secondary antibodies were goat anti-rabbit IgG conjugated with Alexa Fluor 488 (1:500 (v/v); Invitrogen), goat anti-mouse IgG conjugated with Alexa Fluor 594 (1:500 (v/v); Invitrogen) and anti-phalloidin conjugated with Alexa Fluor 594 (1:50 (v/v); Invitrogen).

Primary and secondary antibody incubations varied from 1d to 3d, at 4˚C in agitation.

All images were acquired with a Zeiss LSM 710 confocal microscope with a 20x (dry), 40x (oil) or 63x (oil) objective and treated with ImageJ (FIJI).

Courtship Index

Receptivity videos were used to measure the time the male spent courting, during the first 10 minutes or until copulation. The index was then calculated by dividing the time spent courting by the total time of

observation. Counted were all directly courtship related male actions, including orientation and following.

Locomotion

Single flies were aspirated into arenas and filmed for 5 minutes on a light plate (technical details see receptivity assay). Videos were then analyzed using custom developed software. Output data is distance walked per time interval (mm/minute).

Proboscis Extension / Bloatedness

The bloatedness phenotype was associated with proboscis extension. To assess the quantity of bloated flies, receptivity videos were viewed and every female counted as bloated whenever it had a constantly extended proboscis. The extended proboscis was more reliably discernible, it was either extended or not, while the degree of bloatedness varied.

Egg-Laying

After temperature induced activation of Kir2.1-expression (12 hours at 30˚C; control flies at 18˚C), five females (aged 6-14 days) were allowed to lay eggs on apple agar medium covering a 5 cm plate for 24 hours. After a 6 hour recovery period at 18˚C this procedure was repeated with the same flies (Figure 12).

Counted were only those plates that in the end contained 5 living flies. Data were analyzed using GraphPad software. The statistical test used was the Students t-test.

The number of eggs was calculated as an average: eggs laid per female per experiment.

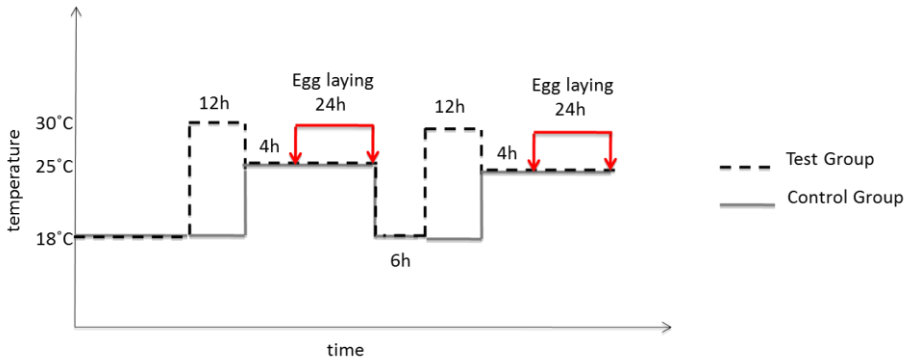

Figure 2.12 Schema of the activation for the egg-laying experiment

Statistical Analyses

Receptivity data was statistically analyzed with the Fisher's exact test in Graph Pad 6.

Locomotion, Courtship Index and egg-laying data was statistically analyzed with the student t-test in Graph Pad 6.

Acknowledgements

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Author Contributions

D.P.H. performed all the experimental work and analyses, with the following exceptions: Image of female reproductive tract (Figure 2.3C) was taken and the specimen prepared by Márcia Aranha; Image of flies in Appendix B was taken by Márcia Aranha; the egg-laying experiment was performed by Sophie Dias. D.P.H. and M.L.V. designed the experiments.

Competing Financial Interests

The authors declare no competing financial interests.

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III. Reduction of the Number of Silenced *apterous* Neurons Using an Intersectional Approach

Summary

In the previous chapter we presented data that identifies *apterous-GAL4* neurons as playing a crucial role in female receptivity behavior. Here we aimed to reduce the number of neurons of interest by employing intersectional strategies. We screened thirteen GAL80-lines and identified two sets of neurons as being involved. In the first set, GAL80-expression rescues receptivity, egg-laying and the proboscis extension/bloatedness-phenotype. This set is *choline acetyltransferase*, *fruitless* and *leucokinin* positive. The second set, which is possibly a subset of the first, is characterized by (partially) rescuing receptivity alone. This second set is additionally *tyrosine hydroxylase*, *glutamic acid decarboxylase* and *cryptochrome* positive. In order to locate these neurons we searched for differences in expression. We directly compared expression patterns by employing brain alignment. However, we were not able to unambiguously identify their location, suggesting that the sought set of neurons is small. In an experiment only silencing *apterous* brain neurons we observed reduced receptivity. In conclusion this work indicates that a

small and novel set of *apterous-GAL4* neurons in the brain is involved in virgin female receptivity.

Introduction

In our previous work we uncovered the involvement of *apterous* neurons in female receptivity. We were able to show that the phenotype – 50% reduced receptivity – resulting from temperature-induced expression of inwardly rectifying potassium channel 2.1 (Kir2.1), is due to *apterous-GAL4* labeled neurons.

Following this we sought to reduce the number of *apterous* neurons in order to identify the set of neurons that is required for this phenotype. Furthermore we aimed to characterize the identity of these neurons and elucidate their relation to courtship relevant stimuli and to the postmating responses. We planned to accomplish this through an intersectional approach using GAL80 lines and the FLIP-OUT technique (Bohm et al., 2010; Lee & Luo, 2001). These techniques create intersections between two sets of cells. The intersection is either excluded from manipulation (GAL80) or is the only set of neurons that is manipulated (FLIP-OUT). Unfortunately we were not able to take advantage of the FLIP-OUT technique. However, the use of various GAL80 lines allowed us to refine the identity of the sought neurons and disentangle the phenotype of reduced receptivity, from reduced egg-laying and the constitutively extended proboscis and bloated abdomen.

Our results indicate that the *apterous-GAL4* line labels two subsets of neurons: One subset controlling female receptivity and another that in addition controls egg-laying, proboscis extension and bloated abdomen. *apterous-GAL4* neurons that specifically control female

receptivity are putative dopaminergic, glutamatergic and cryptochromergic neurons, whereas neurons labeled by *fru-lexA > lexAop-GAL80*, *Cha-GAL80*, *MB-GAL80* and *leucokinin-GAL80*, additionally play a role in egg-laying and proboscis extension/bloatedness. However, an intensive analysis of the anatomy involving brain alignment failed to identify these neurons.

Results

The FLIP-OUT Technique

In this technique two transgene-lines act together to define an intersection in which expression of a third transgene (e.g. GFP) will be driven. One set of cells is defined by a GAL4 line, the other by a FLP-line. Flippase (FLP) is a recombinase that recognizes flippase recognition target (FRT). When using a transgene like UAS-FRT-stop-FRT-effector, the flippase will target the FRT-sites and, by recombination, excise the stop-codon, thereby allowing GAL4 to bind to UAS and drive expression of the effector (e.g. GFP, Kir2.1) in the intersection between the GAL4 line and the FLP line. (Lee & Luo, 2001; Luo, Callaway, & Svoboda, 2008)

We planned to use two FLP-lines, *fru-FLP* (Yu, Kanai, Demir, Jefferis, & Dickson, 2010) because *fruitless (fru)* is involved in the establishment of sexually dimorphic neural circuits, and *MB247-FLP* (Pech et al., 2013) as *apterous-GAL4* labels the mushroom body (MB).

Unfortunately we could not obtain flies with the right genotypes with *fru-FLP*, probably due to adverse interactions of the transgenes. In

order to be able to use this approach, we tested various other ways of setting up the crosses. Additionally we used a P-element transposition of the FLP transgenes to another chromosome. All attempts proved futile.

Intersection of *apterous-GAL4* and *MB247-FLP*

As we will show further down, *MB247-GAL80* rescues the *apterous-GAL4 > UASKir2.1, TubGAL80^{TS}* mediated reduction of receptivity. In order to test the hypothesis, that *apterous* positive MB neurons are responsible for receptivity reduction, we used *MB247-FLP*. In this experiment only neurons that express GAL4 and FLP are silenced by subsequent expression of Kir2.1. If this intersection is responsible for the phenotype we should observe a receptivity reduction.

Our results show that test flies exhibit control levels of receptivity (Figure 3.1) which indicates that neurons in this intersection are not required for the *apterous* phenotype. However, as anatomical analysis revealed (Figure 3.2): The *apterous-GAL4* ∩ *MB247-FLP* intersection of this experiment is rather small and weak. Of the MB structures only the γ-lobe is discernible. *apterous-GAL4* in contrast strongly labels the Kenyon cells and the α-, β- and γ-lobes. Furthermore *MB247-GAL80* completely inhibits *apterous-GAL4* driven GFP expression in all MB structures.

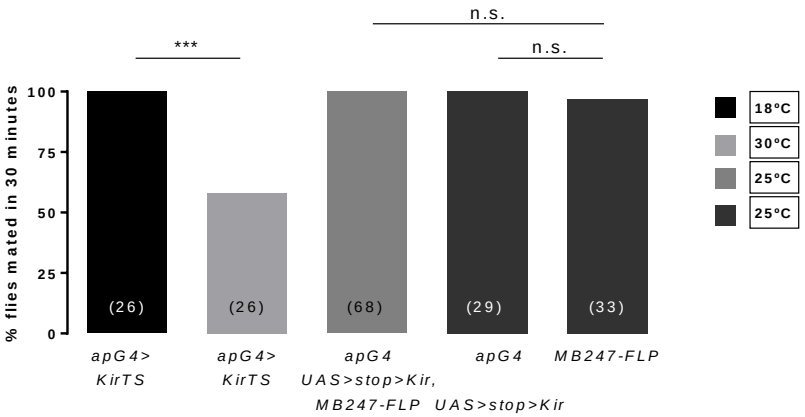

Figure 3.1 Kir2.1 expression in the intersection of *apterous-GAL4* and *MB247-FLP* does not reduce receptivity

First two bars: Receptivity of *apterous-GAL4 > UASKir2.1. TubGAL80^{TS}* plotted as cumulative percentage of copulation events across couples, during 30 minutes. Control conditions in black, experimental conditions in grey. The remaining bars show the receptivity of flies of the indicated genotypes kept at 25˚C. Sample sizes are indicated in brackets on the respective column. *p<0.05, **p<0.01 ***p<0.001***p<0.0001, n.s.=not significant, Fisher's exact test. *apG4=apterous-GAL4; Kir^{TS}=UASKirTS, TubGAL80^{TS}*

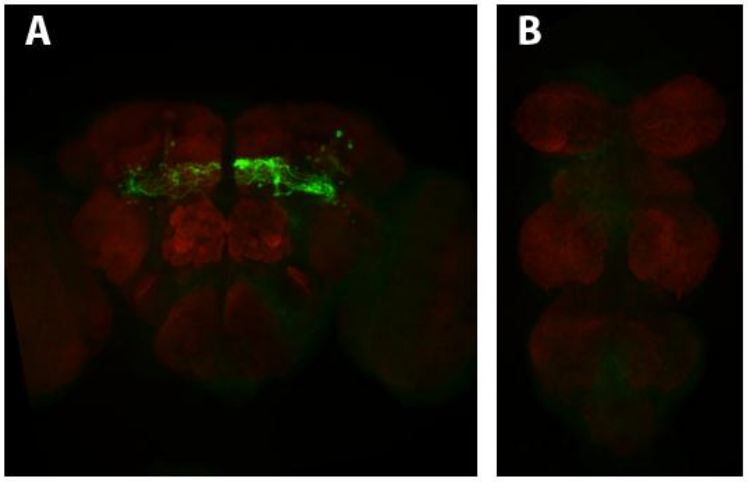

Figure 3.2 Intersection of *apterous-GAL4* and *MB247-FLP*
Representative images of brain and VNC of flies carrying *apterous-GAL4*, *MB247-FLP* and *UAS > stop > mCD8-GFP* transgenes. The amount of Kenyon cells labeled is smaller than the one found in flies with the *apterous-GAL4 ∩ MB247-GAL4* intersection. In addition, the strength of labeling is low. Neuropil in red (NC82). Neurons of intersection in green (mCD8-GFP).

Screen of GAL80 Lines

In order to reduce the number of neurons that could be responsible for the reduction of receptivity upon silencing with Kir2.1 we continued with another intersectional strategy using GAL80 lines. This strategy is based on the exclusion of GAL80-expressing neurons from the set of GAL4-expressing neurons. This is achieved by GAL80-mediated inhibition of GAL4 activity. In the neurons expressing GAL80, *apterous-GAL4* will not be able to drive expression of Kir2.1.

If flies carrying *apterous-GAL4* and *UASKir2.1, TubGAL80^{TS}* and a particular GAL80 line exhibit the same receptivity as flies carrying only *apterous-GAL4* and *UASKir2.1, TubGAL80^{TS}* the conclusion will be that the two lines intersect in neurons that are not required for receptivity or that do not intersect at all. If, on the other hand, we

observe a rescue of receptivity to wild-type levels, we will infer that neurons in the intersection are likely to be required for receptivity.

To this end we performed a screen with the following 13 GAL80-lines (Figure 3.3). *Cha3.3kb-GAL80* (Acebes, Martín-Peña, Chevalier, & Ferrús, 2011; Kitamoto, 2002), *GAD-GAL80* (Sakai, Kasuya, Kitamoto, & Aigaki, 2009) and *TH-GAL80* (Sitaraman et al., 2008) are lines that label neurotransmitter expressing neurons, i.e. cholinergic neurons (acetylcholine via choline acetyl-transferase, Cha(t)), gabaergic neurons (γ-aminobutyric acid (GABA), via glutamic acid decarboxylase, GAD) and dopaminergic neurons (dopamine via tyrosine hydroxylase, TH). The line *cry-GAL80* expresses GAL80 in cells expressing the circadian photoreceptor cryptochrome, which is involved in circadian rhythms (Stoleru, Peng, Agosto, & Rosbash, 2004); *lk-GAL80* labels leucokinin neurons that intersect with *apterous* neurons (Herrero, Magariños, Torroja, & Canal, 2003). Using the LexA/lexAop-system (works analogously to the GAL4/UAS-system) we could access *fruitless* neurons via *FruP1lexA > lexAop-GAL80*. The line *CCAP-GAL80* allows access to cells expressing the neuropeptide crustacean cardioactive peptide which is implied in timing of ecdysis (molting; Park et al. 2004); *MB247-GAL80* labels mostly the mushroom body (Krashes, Keene, Leung, Armstrong, & Waddell, 2007); *Tsh-GAL80* is widely expressed in the VNC but not the brain (Clyne & Miesenböck, 2008); *svp-GAL80*, short for seven up, labels oenocytes (Gutierrez, Wiggins, Fielding, & Gould, 2006) and *EHups-GAL80* drives GAL80 expression in cells that express the neuropeptide, eclosion hormone (EH), a key regulator of ecdysis (McNabb, Baker, & Agapite, 1997). *C,D,E,F/eve-GAL80* acts as transcriptional repressor controlling segmentation during embryonic development (Han & Manley, 1993). Lastly, *ppk-GAL80*, short for

pickpocket, expresses GAL80 in mechanosensory neurons but importantly was also shown to drive GAL80 expression in sex peptide sensory neurons (SPSN) in the uterus (Häsemeyer, Yapici, Heberlein, & Dickson, 2009; Yang et al., 2009).

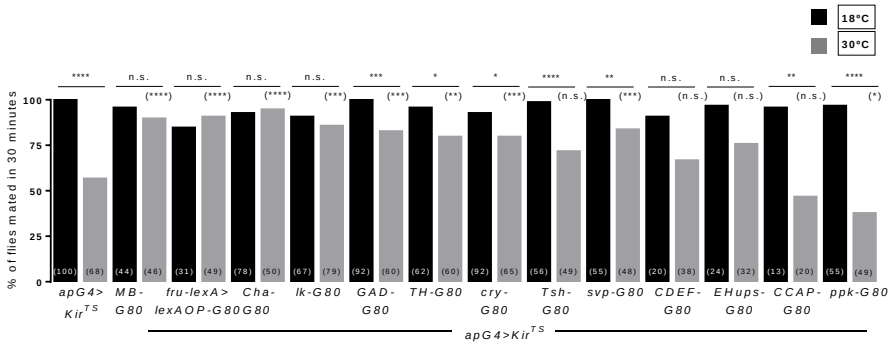

Figure 3.3 Receptivity levels of *apterous-GAL4* lines after neuronal subtraction using different GAL80 lines
Receptivity of *apterous-GAL4 > UASKir2.1, TubGAL80^{TS}* in combination with the respective GAL80 lines, plotted as cumulative percentage of copulation events across couples during 30 minutes. Control condition is in black, experimental condition in grey. Sample sizes are indicated in brackets on the respective column: *p<0.05, **p<0.01 ***p<0.001***p<0.0001, n.s.=not significant, Fisher's exact test. Signs in brackets denote comparison between *apterous-GAL4*, *UASKir2.1. TubGAL80^{TS}* at 30˚C and the respective condition. *apG4>Kir^{TS}=apterous-GAL4 > UASKir2.1, TubGAL80^{TS}; G80=GAL80*

For a full rescue, receptivity had to meet two criteria: 1. Being statistically different from the receptivity of flies with all *apterous* neurons silenced. 2. Being not statistically different from the temperature control. Four genotypic combinations displayed a full rescue: *Cha-GAL80*, *apterous-GAL4 > UASKir2.1, TubGAL80^{TS}* (p=0.7359, vs. respective control and p<0.0001 vs. *apterous-GAL4 > UASKir2.1, TubGAL80^{TS}* at 30˚C), *lk-GAL80*, *apterous-GAL4 > UASKir2.1, TubGAL80^{TS}* (p=0.4648 vs. respective 18˚C control, and p<0.0001 vs. *apterous-GAL4 > UASKir2.1, TubGAL80^{TS}* at 30˚C), *MB-GAL80*, *apterous-GAL4 > UASKir2.1, TubGAL80^{TS}* (p=0.4349 vs. respective 18˚C control, and p<0.0001 vs. *apterous-GAL4 >*

UASKir2.1, TubGAL80^{TS} at 30˚C) and *frulexA > lexAop-GAL80*, *apterous-GAL4 > UASKir2.1, TubGAL80^{TS}* (p=0.4983 vs. respective 18˚C control, and p<0.0001 vs *apterous-GAL4 > UASKir2.1, TubGAL80^{TS}* at 30˚C). Interestingly, three of these lines (*frulexA > lexAop-GAL80*, *Cha-GAL80* and *lk-GAL80*) also resulted in full rescues when tested for egg-laying and the proboscis extension (Figure 3.4 and Figure 3.5), suggesting that the intersection of each of these lines with *apterous-GAL4* includes neurons that are required for all three phenotypes.

Three lines did not meet either of the two criteria and thus did not rescue receptivity: *CCAP-GAL80*, *apterous-GAL4 > UASKir2.1, TubGAL80^{TS}* (p=0.0026 vs. respective 18˚C control; p=0.5624 vs. *apterous-GAL4 > UASKir2.1, TubGAL80^{TS}* at 30˚C), *ppk-GAL80*, *apterous-GAL4 > UASKir2.1, TubGAL80^{TS}* (p<0.0001 vs. respective 18˚C control; p=0.0362 vs. *apterous-GAL4 > UASKir2.1, TubGAL80^{TS}* at 30˚C; this difference results from a more strongly reduced receptivity than *apterous-GAL4 > UASKir2.1, TubGAL80^{TS}* at 30˚C and thus is not to be considered as rescue effect) and *Tsh-GAL80*, *apterous-GAL4 > UASKir2.1, TubGAL80^{TS}* (p<0.0001 vs. respective 18˚C control; p=0.0764 vs. *apterous-GAL4 > UASKir2.1, TubGAL80^{TS}* at 30˚C). This result strongly suggests that these lines do not intersect with *apterous-GAL4*, or that the intersection does not contain neurons that are involved in receptivity. The lack of rescue with *ppk-GAL80* also presents further evidence that *apterous-GAL4* does not intersect with the sex peptide sensory neurons described by Häsemeyer et al. (2009) and Yang et al. (2009). As these lines do not rescue receptivity they were not included in the subsequent experiments, with the exception of *Tsh-GAL80*, as we reasoned that

apterous neurons in control of egg-laying are likely to be located in the VNC.

The receptivity results obtained with the lines *EHups-GAL80* and *CDEF-GAL80* are inconclusive as they only meet the second of the two criteria: they are not statistically different from the temperature control. This might be explained by low n, which in turn was due to low offspring numbers and sickly appearing flies. Thus these lines were not tested for egg-laying and proboscis extension.

The four lines *TH-GAL80*, *GAD-GAL80*, *cry-GAL80 and svp-GAL80* show only partial rescues of receptivity as they only meet the first criterion, being statistically different from the receptivity of flies with all *apterous* neurons silenced: *TH-GAL80* (to 18˚C, p=0.0139 and to *apterous-GAL4 > UASKir2.1, TubGAL80^{TS}* at 30˚C p<0.0038), *GAD-GAL80* (to 18˚C, p=0.0001 and to *apterous-GAL4 > UASKir2.1, TubGAL80^{TS}* at 30˚C p<0.0002), *cry-GAL80* (to 18˚C, p=0.0410 and to *apterous-GAL4 > UASKir2.1, TubGAL80^{TS}* at 30˚C p<0.0007) and *svp-GAL80* (to 18˚C, p=0.0016 and to *apterous-GAL4 > UASKir2.1, TubGAL80^{TS}* at 30˚C p<0.0010). However the differences to their respective temperature controls are almost not significant, especially of *TH-GAL80* and *cry-GAL80*, while the difference to the positive control (*apterous-GAL4 > UASKir2.1, TubGAL80^{TS}* at 30˚C) is very significant in all four. We decided to test *TH-GAL80*, *GAD-GAL80* and *cry-GAL80* for egg-laying and proboscis extension/bloatedness. We excluded, however, *svp-GAL80* as crosses yielded low numbers of progeny.

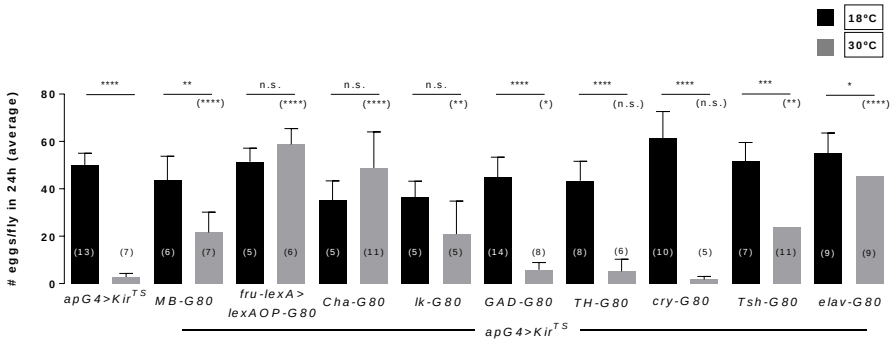

Figure 3.4 Egg-laying screen of several GAL80 lines in combination with silenced apterous neurons
Egg-laying of *apterous-GAL4 > UASKir2.1. TubGAL80^{TS}* in combination with the respective GAL80 lines, plotted as the average of eggs laid per fly per 24 hours. Control condition is in black, experimental condition in grey. All females were mated before the experiment. Sample sizes are indicated in brackets on the respective column (n of 1 equals one plate with 5 flies): *p<0.05, **p<0.01 ***p<0.001***p<0.0001, n.s.=not significant, student t-test. Signs in brackets denote comparison between *apterous-GAL4*, *UASKir2.1. TubGAL80^{TS}* at 30˚C and the respective condition. *apG4>Kir^{TS}=apterous-GAL4 > UASKir2.1, TubGAL80^{TS}; G80=GAL80*

Evaluation of Egg-laying and Proboscis Extension/Bloatedness

Analysis of the egg-laying and proboscis extension/bloatedness, revealed that *TH-GAL80*, *GAD-GAL80* and *cry-GAL80* do not rescue these two phenotypes, not even partially. This is a very interesting result, as it suggests that the role of neurons in the respective intersections in receptivity is dissociable from egg-laying and proboscis extension/bloatedness. The intersection created with *fru-lexA > lexAop-GAL80*, *Cha-GAL80 and lk-Gal80* on the other hand show full rescues for egg-laying and proboscis extension/bloatedness as well as for receptivity, whereas *MB-GAL80* and *Tsh-GAL80* only effect partial rescues.

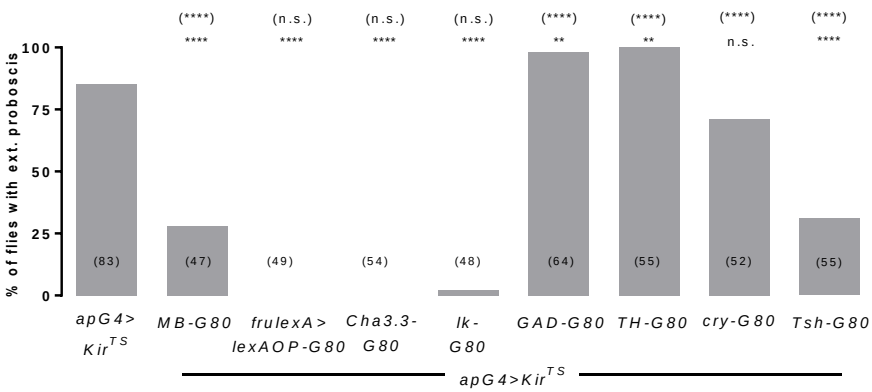

Figure 3.5 Proboscis extension/bloatedness of GAL80 lines in combination with silenced apterous neurons
Proboscis extension of *apterous-GAL4 > UASKir2.1. TubGAL80^{TS}* in combination with the respective GAL80 lines, plotted as the percentage of flies with an extended proboscis. Videos of the receptivity screen were used. Sample sizes are indicated in brackets on the respective column *p<0.05, **p<0.01, ***p<0.001***, p<0.0001, n.s.=not significant, Fisher's exact test. Signs without brackets denote comparison between *apterous-GAL4 > UASKir2.1. TubGAL80^{TS}* at 30˚C and the respective condition. Signs in brackets denote comparison between *apterous-GAL4*, *UASKir2.1. TubGAL80TS* at 18˚C and the respective condition. *apG4>Kir^{TS}=apterous-GAL4 > UASKir2.1, TubGAL80^{TS}; G80=GAL80*

Anatomical Analysis of GAL80 line ∩ *apterous-GAL4* Intersections

The series of experiments described in the preceding section, offer insights into the possible identity of the neurons responsible for the *apterous* phenotypes. The experiments suggest the existence of intersections that are made up of two sets of neurons. Possibly one set is a subset of the other. The intersections of *lk-GAL80* and *MB-GAL80* and especially *Cha-GAL80* and *fru-lexA > lexAop-GAL80* with *apterous-GAL4* are possibly larger intersections, including neurons responsible for receptivity, egg-laying and proboscis extension/bloatedness, whereas the intersections of *TH-GAL80*, *GAD-GAL80* and *cry-GAL80* with *apterous-GAL4* appear to be only

required for receptivity. Potentially these are smaller intersections with fewer neurons.

In order to identify these intersections the expression patterns of brains with a GAL80 line present, have to be compared with the expression pattern of *apterous-GAL4 > UASKir2.1, TubGAL80^{TS}* only brains. However no brain is quite like the other: small differences in size, differences in the orientation during imaging and distortions introduced during dissection, fixation and mounting are the rule. Due to these differences a superimposition of two brains and direct comparison in the search for differences in the expression pattern is not feasible.

To overcome this limitation and investigate the neuroanatomical nature of these intersections, we decided to take advantage of a brain alignment method that compensates and corrects these differences. We used the CMTK toolkit: http://www.nitrc.org/projects/cmtk (Jefferis et al., 2007; Rohlfing & Maurer, 2003). It consists of various algorithms that deform and move around an image and its different areas with the aim to fit it to a standard image (in our case brain and VNC). This will compensate distortions introduced by development and the staining procedure and render brains (and VNCs) comparable. Aligned images can then be superimposed to each other which facilitates the comparison of the expression pattern. A standard brain can be one exemplary brain or the average of many brains that were aligned to an exemplary brain (or VNC). For this the neuropil channel is used (NC82). We chose to create a standard brain out of the average of ~20 aligned brains. Shown in Figure 3.6A, B and C are the standard image stacks we used to align all brain images to.

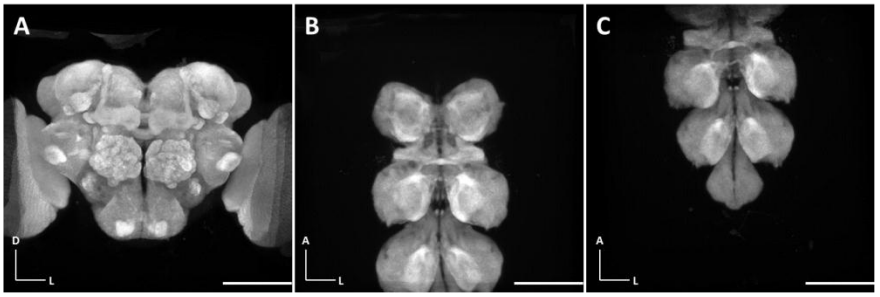

Figure 3.6 Z-projection of the standard brain stacks used for brain alignment. **(A, B, C)** Images of the average of ~20 aligned brains and VNCs; brain **(A)**, prothoracic and mesothoracic ganglion **(B)**, metathoracic and abdominal ganglion **(C)**. Each image represents the z-projection of the average of several dozens of samples that aligned best to one particular brain (NC82; see Material and Methods for detailed description). Scale bars, 100µm.

For this anatomical analysis we used the same GAL80 lines that we used in the preceding experiments – in this case we drove expression of mCD8-GFP instead of Kir2.1 - and imposed each aligned image onto an aligned image of an *apterous-GAL4 > UAS-mCD8-GFP* fly (Figure 3.7 shows such a superposition). The alignment works well, allowing for tracing of larger tracts and processes (Figure 3.7 arrow); only if the innervation gets too complex and fine-threaded comparing these areas is not feasible anymore (Figure 3.7 arrowhead). In this manner, differences of expression between the two images can be detectable. As a result of the nature of the expression pattern of *apterous-GAL4* however, which contains many neurons and larger areas of innervation with very fine processes, this was not straightforward. A displacement of cell bodies in the course of the dissection procedure can occur and brain alignment cannot correct for this, as it is the neuropil channel that is aligned to a standard neuropil. The cell bodies are outside of the neuropil and do not carry information.

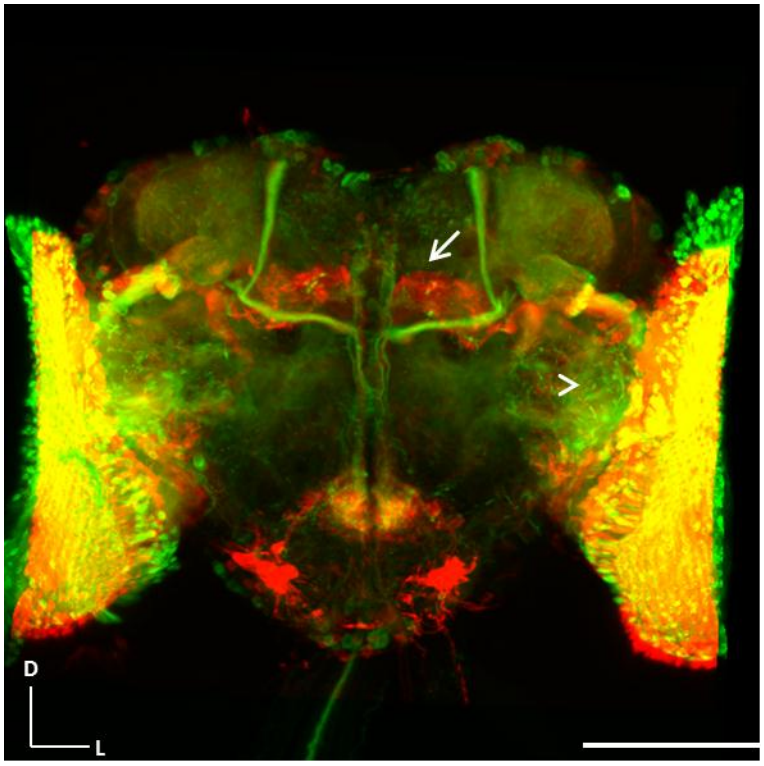

Figure 3.7 Example of a superposition
Brain expressing *apterous-GAL4* driven GFP in red. Brain with inhibition of GAL4 by *Cha-GAL80* in green. Both were aligned to the standard brain. Overlap between the two images is seen in orange. Areas with green or red dominance indicate difference in expression (MB γ-lobes; arrow), or fluctuations in intensity of labeling (arrowhead). Scale Bar, 100µm.

The *apterous-GAL4* Expression Pattern

In the course of this analysis it became clear that the complexity of the *apterous-GAL4* expression pattern required a more detailed description in order to understand what is labeled by *apterous-GAL4* and what is not. Therefore we will present here a detailed description of the *apterous-GAL4* expression pattern.

In order to facilitate orientation we schematized the *apterous-GAL4* expression pattern (Figures 3.8B, 3.8D, 3.9B, 3.10B and 3.10D). We depict areas of neuron clustering as well as single neurons that were repeatedly locatable across several brains and VNCs. We do not depict the intense labeling of cells and processes of the optic lobes or forming the mushroom bodies.

The Brain

Cell bodies located in the optic lobes send projections into the superior brain, where they bundle and then form the optic tubercle (Figure 3.8). Kenyon cells from the superior-posterior brain form the structures comprising the MB; the γ-lobe, the α- and β-lobes and to a lesser extent the α´- and β´-lobes. Scattered and inconsistently placed cell bodies can be found between protocerebrum and optic lobes. The ventrolateral protocerebrum (VLP) is finely innervated, exhibiting stronger labeling in its inferior part. Also the lateral horn, the superior medial and the superior lateral protocerebrum receive some fine innervations. However, completely free of innervation are the areas of the antennal lobes and the AMMC.

On the dorsal side we see the tightly clustered Kenyon cells (KC), that are surrounded by an area of scattered cell bodies (Figure 3.8D, cluster *1*). Two further clusters contain more tightly packed neurons. One is placed along the superior midline (Figure 3.8D, cluster *3*), the other is located lateral of the periesophageal foramen (Figure 3.8D, cluster *2*).

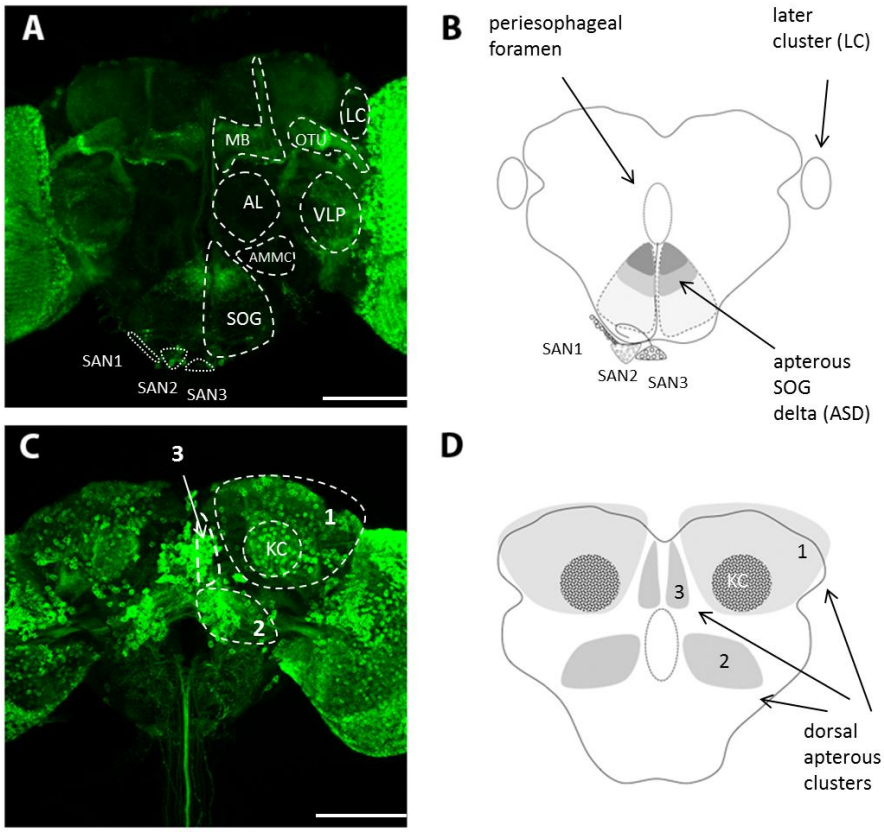

Figure 3.8 Representative image of an *apterous-GAL4* > *UAS-mCD8-GFP* brain and schema
(A) z-projection of the anterior view of the brain, **(C)** z-projection of the posterior view of the brain. Schema of anterior (B) and posterior **(D)** areas labeled by *apterous-GAL4*; grey shaded areas roughly represent intensity of innervation; SOG *apterous* neurons (SAN). Scale bars, 100µm.

The SOG

The SOG is globally and finely innervated, to a degree that allows its shape to be clearly discernible. Its superior part is more strongly innervated and more strongly labeled; we will from here on refer to it as: apterous SOG delta (ASD; Figure 3.8A and B). The ASD, which roughly corresponds to the prow (PRW; Ito et al. 2014) is divided into a more intensely labeled superior delta and a less intensely labeled

inferior band. The cell bodies providing these processes are to a large part the ones that are positioned along the inferior border of the SOG.

The innervations in the superior part join, and project upwards as the median bundle. There are three clusters around the floor of the SOG that we number 1 to 3, from lateral to medial and will refer to as SOG *apterous* neurons (SAN1, SAN2 and SAN3). SAN1 (3-20 medium sized neurons) and SAN2 (~20 small neurons) bundle together to form a process and project upwards to the esophageal foramen. The remaining cluster SAN 3 (~10 medium sized neurons) innervates the SOG.

The VNC

Generally all VNC ganglia bear some global innervation, which then join to form tracts along the midline. These tracts project through the cervical connective (CvC). Furthermore, large, rather loose bundles of fibers exit each ganglion ventrally (except for the abdominal ganglion).

Pro- and Mesothoracic Ganglion

The prothoracic ganglion has one (bilateral) area, populated with neurons, of small size, that starts superior medially and continues around the curve of the ganglion to end posterior laterally. The shape depicted in the schema (Figure 3.9) is that of an x formed by two thick half circles. In this area small neurons can be found continuously, yet sometimes more densely clustered.

The *apterous* expression pattern in the mesothoracic ganglion is similar to that in the prothoracic ganglion. The occurrence of neurons in the x-shaped area is more uniform, with one big and bright cluster

at the anterior end of the half circle that contains medium-sized neurons.

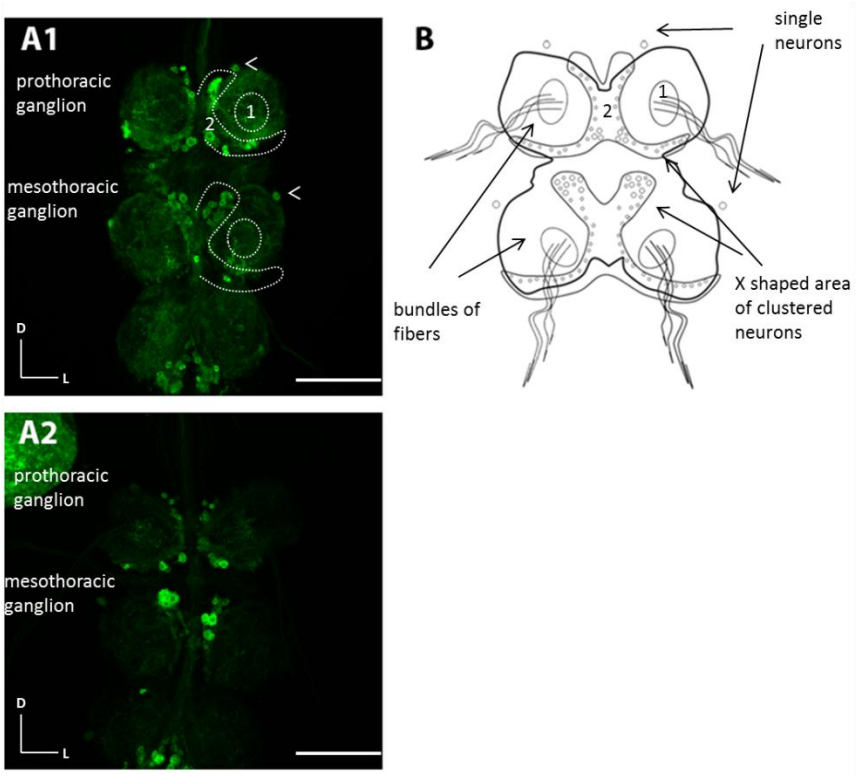

Figure 3.9 Two examples of images of *apterous-GAL4 > UAS-mCD8-GFP* labeled prothoracic and mesothoracic ganglia and schema
(A1, A2) Apparent is the fluctuation in staining intensity and location of neurons within clusters. Ganglia shape is clearly discernible by the fine, widespread innervation. **(C)** Circle size and number is roughly representative of neuron size, and occurrence (depicted are significantly less neurons, except for the four big ones that are depicted outside of the ganglia outline.). Scale bar, 100µm.

The dorsal half of these two ganglia is devoid of cell bodies. There are only four big cells outside of the described areas. Two, one on each side, are located at the anterior end of the prothoracic ganglion. Two more, one on each side, are located laterally of the mesothoracic ganglion, often towards the anterior. These four neurons are located dorsally of the x-shaped areas.

Metathoracic and Abdominal Ganglion

In the metathoracic ganglion two cell clusters are positioned posterior medially, at about the same depth, ventrally (Figure 3.10). The smaller cluster contains medium-sized neurons and the bigger one small neurons. All of them seem to feed into fiber bundles projecting to the midline, following it upwards.

Several individual neurons can be encountered on the ventral and the dorsal half of both the metathoracic and the abdominal ganglion. We depicted them to the best of our knowledge in the respective schema (Figure 3.10).

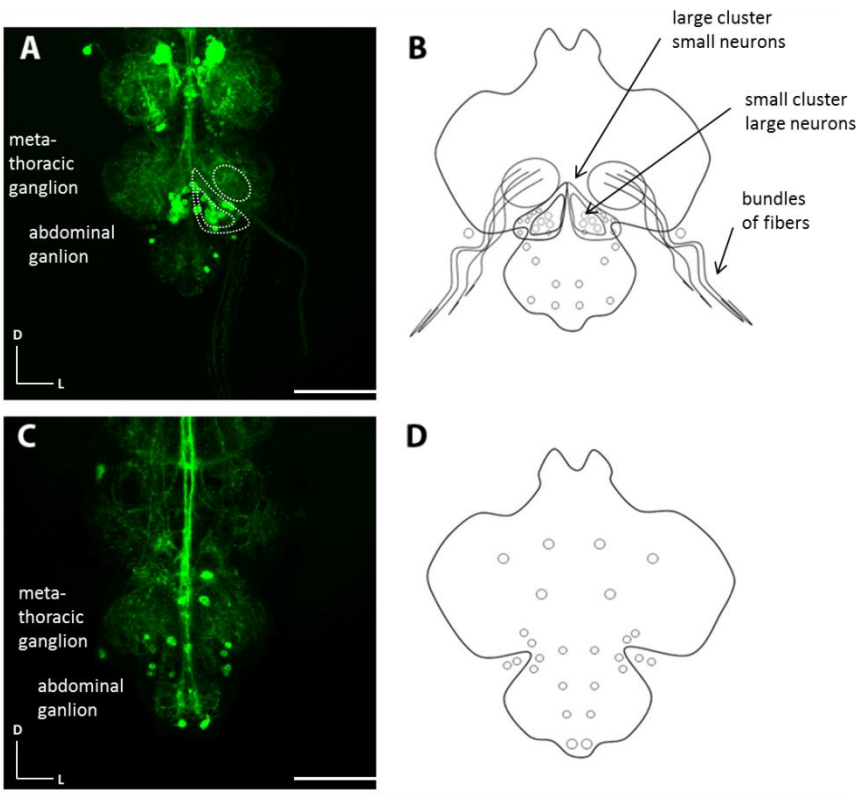

Figure 3.10 Example of an *apterous-GAL4* > *UAS-mCD8-GFP* labeled metathoracic and abdominal ganglion and schema
(A) z-projection of a whole stack, *apterous-GAL4* > *UAS-mCD8-GFP* VNC, anterior view. **(C)** z-projection of the dorsal half, *apterous-GAL4* > *UAS-mCD8-GFP* with *TH-GAL80* (this particular VNC looked best as a representative stack of dorsal *apterous* neurons). Compared to the schema several neurons are missing or at a slightly different location. Only looking at many brain stacks the whole picture will assemble. **(C)** Ventral *apterous* labeled areas and neurons. Kidney shapes outline tightly packed cell clusters; depicted are less neurons. Circles outside of these clusters correspond to the observed number of neurons. **(D)** Dorsal *apterous* labeled neurons; Circles correspond to the observed number. Circle size is roughly representative of neuron size. Scale bars, 100µm.

The Result

After thorough examination of brains and VNCs of flies carrying *apterous-GAL4* and *UAS-mCD8-GFP* and subsequent comparison to brains and VNCs of flies carrying *apterous-GAL4*, *UAS-mCD8-GFP*

and a GAL80 line (see Appendix C) we came to the conclusion that there is no reliably discernible difference in expression between any of the brains or VNCs. We cannot find any neurons that are reliably present in *apterous-GAL4 > UAS-mCD8-GFP* tissue that are not in the tissue where GAL80 is expressed. However, there are a few restrictions to this statement.

Individual Descriptions of the *apterous-GAL4* Expression Pattern in combination with GAL80 Lines

MB247-GAL80 and lk-GAL80

As described above receptivity was fully rescued with *MB-GAL80* and *lk-GAL80*. Rescues of egg-laying and proboscis extension were partial. What insight does the comparison of *apterous-GAL4 > UAS-mCD8-GFP* and *MB-GAL80*, or *apterous-GAL4 > UAS-mCD8-GFP* and *lk-GAL80* yield?

The MB is not observable in the *MB247-GAL80* flies. Additionally we spotted a difference in expression in the ASD (Figure 3.8A and B). The less strongly labeled inferior band is missing in *MB-GAL80* brains.

In the *lk-GAL80* brains the MB γ-lobe is the only structure where we notice change in expression. In all of the four brains that we examined, the γ-lobe seemed to be less widely labeled and less strongly labeled, than in any of the *apterous-GAL4 > UASKir2.1, TubGAL80^{TS}* brains. Interestingly, there was a strong variability as to the degree of diminishment in the *lk-GAL80* brains. In one brain we could not discern any γ-lobe labeling.

Cha-GAL80 and fru-lexA > lexAop-GAL80

In the brains of these two genotypes, the MB continued to be the only structure we were able to encounter that showed clearly noticeable diminishment of GFP expression. In *Cha-GAL80* specimen, the labeling of the γ-lobe was reduced. While in *fru-lexA > lexAop-GAL80* specimen parts of the γ-lobes were the only MB structures that retained some labeling.

Silencing of *lk-GAL4* and *OK107-GAL4*

The two lines *lk-GAL80* and *MB247-GAL80* each yielded a rescue. This implies the possibility that the respective GAL4 lines, *lk-GAL4* and *MB247-GAL4*, label receptivity-related neurons that when silenced reduce receptivity. In addition *lk-GAL4* labels few neurons and *MB247-GAL4* labels few neurons other than the MB. We silenced each line to address whether this triggers reduced receptivity as observed when silencing *apterous-GAL4* neurons.

MB neurons were assessed using *OK107-GAL4* instead of *MB247-GAL4*, since flies of this line crossed to *UASKir2.1, TubGAL80^{TS}* yielded non-viable progeny presumably due to expression in glia. To our surprise silencing either *lk-GAL4* neurons or *OK107-GAL4* neurons individually did not affect female receptivity (Figure 3.11). We note that we confirm the observations of Cognini et al. (2011): A bloated abdomen and an extended proboscis after Kir2.1 expression in *lk-GAL4* neurons; probably due to liquid retention (Cognigni, Bailey, & Miguel-Aliaga, 2011).

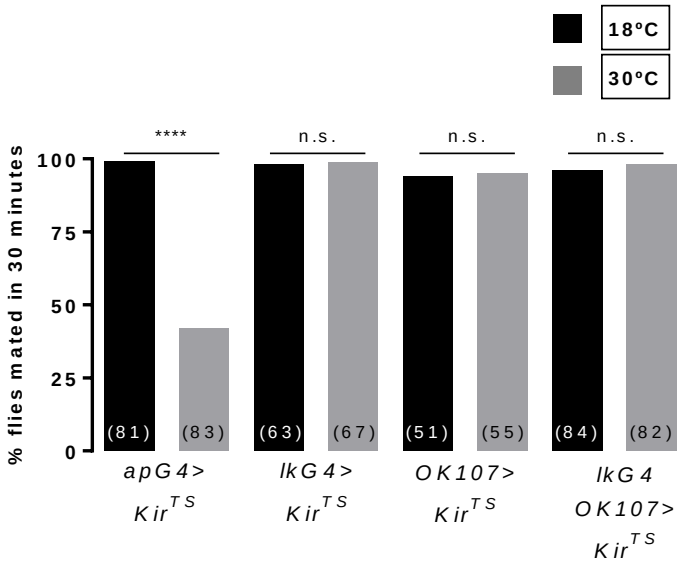

Figure 3.11 Simultaneous silencing of *lk-GAL4* and *OK107-GAL4* does not reduce receptivity
Receptivity of silenced *leucokinin-GAL4* and *OK107-GAL4* flies as well as of flies with simultaneously silenced *lk-GAL* and *OK107-GAL4* neurons, plotted as cumulative percentage of copulation events across couples, during 30 minutes. Control condition is in black, experimental condition in grey. Sample sizes are indicated in brackets on the respective column; *p<0.05, **p<0.01 ***p<0.001***p<0.0001, n.s.=not significant, Fisher's exact test. *apG4>Kir^{TS}=apterous-GAL4 > UASKir2.1, TubGAL80^{TS}; G4=GAL4*

These results led us to speculate about possible explanations. The results could be explained by the existence of two, individually sufficient pathways that receptivity signals can take. When we silence one of them, the other is still active, and can compensate the silencing effect and provide for wild-type behavior. We tested this hypothesis by simultaneously silencing both lines: *lk-GAL4* and *OK107-GAL4*. Our results show no effect on receptivity and thus reject our hypothesis: activity in neurons labeled by these lines is not required for receptivity.

Discussion

As we have shown in the preceding Chapter II, *apterous* positive cells are involved in the female's decision whether to accept a courting male for copulation or not. a*pterous-GAL4* however, labels several neurons in many parts of the nervous system which did not allow us to identify the phenotype-effecting cluster. Furthermore, silencing of those neurons entails two additional phenotypes: reduced egg-laying and continuous proboscis extension in addition to a bloated abdomen.

Aiming to reduce the set of neurons involved in the receptivity phenotype we chose an intersectional approach. First we worked on the FLIP-OUT technique, in which transgene-action is confined to the intersection. This bears the powerful possibility of finding an intersection of neurons that, when silenced, affects receptivity – and only receptivity. However, the *fru-FLP* transgene did not yield viable offspring in combination with *apterous-GAL4* and *UAS > stop > mCD8-GFP*, probably due to genetic interactions. Silencing neurons in the intersection between *MB247-FLP* and *apterous-GAL4* did not reduce receptivity, indicating that neurons in this intersection are not required for receptivity. The amount of neurons in the intersection however, appeared to be smaller than the amount of neurons labeled in the MB by *apterous-GAL4*.

Thus we focused our efforts on a variation of the intersectional approach that uses GAL80 lines. In a series of experiments we found several GAL80 lines that express in *apterous* cells and rescue the *apterous* phenotypes.

Our results indicate that there is a set of neurons that is involved in all three observed phenotypes. These neurons are likely to be *fru* positive, *Cha* positive, *leucokinin* positive and *MB247* positive. The latter only seems to be partially required for the egg-laying phenotype, as GAL80 expression in the respective intersection only yielded partial rescues of egg-laying. Interestingly however, removing silencing in the intersections created by *apterous-GAL4* and the lines *TH-GAL80*, *GAD-GAL80* or *cry-GAL80* rescues only receptivity, strongly indicating, that these three intersections in fact contain neurons required for receptivity.

Where are these neurons? To which structure do they belong? In the attempt to answer this question we performed the same type of experiment, but now expressing GFP instead of Kir2.1. We then aligned the obtained images to a standard brain and compared them to images of brains (that were equally aligned) expressing GFP in all *apterous* cells.

Following this procedure we found that the only structures that reliably disappear in GAL80-expressing brains are parts of the MB (especially in *frulexA > lexAop-GAL80*, *Cha-GAL80*, *lk-GAL80* and *MB-GAL80*). This was rather unexpected as a previous study had shown that ablation of the MB does not affect receptivity (Fleischmann & Cotton, 2001; Kido & Ito, 2002). Further three points can be noted: 1. The intersections are likely to be rather small, as we cannot safely say that we find disappearing neurons (apart from the Kenyon cells). 2. Several of the *apterous* labeled cell clusters are tightly packed with large amounts of cells. We cannot exclude the possibility that we miss differences in expression within clusters. 3. Most cell clusters are anatomically positioned in a way that makes them prone to be partly dissected away, which may explain why size

and shape of clusters, and the number of neurons they contain was variable from brain to brain. This was especially the case for the three clusters below the SOG (SAN1, 2 and 3).

Our results from experiments silencing *OK107-GAL4* and *lk-GAL4*, either individually or simultaneously, suggest that neither of the labeled neurons are required for receptivity. Thereby we confirm the results of earlier studies that the MB is not required for receptivity (Fleischmann & Cotton, 2001; Kido & Ito, 2002). Moreover we confirm with these results that a bloated abdomen and an extended proboscis, probably due to liquid retention (Cognigni et al., 2011), are not causing reduced female receptivity.

The presented data strongly indicates that there is a set of *apterous* neurons that is defined by co-expression of *fru-lexA > lexAop-GAL80*, *Cha-GAL80*, *MB247-GAL80* and *lk-GAL80*, which is responsible for all three *apterous* phenotypes. Furthermore our results indicate the existence of a subset of neurons that is only responsible for reduced receptivity. This set is defined by co-expression of *TH-GAL80*, *GAD-GAL80* and *cry-GAL80*. These *apterous* receptivity neurons (ARNs) are either not part of the PMRs, as unaffected egg-laying in virgins with silenced *apterous* neurons suggest, or are located further downstream of the pathway beyond a hypothetical bifurcation that separates PMRs.

This work provides insight into the identity of neurons that are involved in receptivity. It establishes the existence of a potentially small set of *apterous-GAL4* neurons that is characterized by co-expression of *TH-GAL80*, *GAD-GAL80* and *cry-GAL80*. Further efforts may use this information to locate these neurons and describe

their connectivity and function within female *Drosophila* courtship behavior.

Materials and Methods

Fly Stocks

Flies were reared on a standard medium at 18ºC in a 12 hour light / 12 hour dark cycle.

Stock List

apterous-GAL4 (Stevens & Bryant, 1985).

C,D,E,F/eve-GAL80 (Han & Manley, 1993).

CCAP-GAL80 (Park et al., 2004);

Cha3.3kb-GAL80 (Acebes et al. 2011),

Cry-GAL80 (Stoleru et al., 2004);

EHups-GAL80 (McNabb et al., 1997).

fru-FLP (Yu et al., 2010)

FruP1lexA (Mellert, Knapp, Manoli, Meissner, & Baker, 2010)

GAD-GAL80 (Sakai et al., 2009),

Kir2.1 (Baines & Uhler, 2001)

lexAop-GAL80 (Thistle, Cameron, Ghorayshi, Dennison, & Scott, 2012),

lk-GAL80 (Herrero et al., 2003).

MB247-FLP (Pech et al., 2013)

MB247-GAL80 (Krashes et al., 2007),

ppk-GAL80 (Yang et al., 2009).

svp-GAL80 (Gutierrez et al., 2006)

TH-GAL80 1c (Sitaraman et al., 2008).

Tsh-GAL80 (Clyne & Miesenböck, 2008);

TubGAL80^{TS} (McGuire, Le, Osborn, Matsumoto, & Davis, 2003)

UAS-mCD8-GFP (Lee & Luo, 1999)

Neural Manipulation and Receptivity Assay

We used a temperature-inducible genetic system to silence specific sets of neurons in the adult female and then tested the effect of neural silencing on female receptivity (Baines & Uhler, 2001). Neuronal manipulation was achieved by the expression of Kir2.1, an inwardly rectifying potassium channel whose expression causes hyperpolarization of the neurons (Baines & Uhler, 2001). Kir2.1 expression was targeted to subsets of cells using the GAL4/UAS system (Brand & Perrimon, 1993), which utilizes the yeast derived transcription activator protein GAL4 and its target sequence, the "upstream activating sequence" (UAS). Insertion of GAL4 into the Drosophila genome (possible by different means) in front of a driver leads to the expression of GAL4 in a defined set of cells. If an engineered sequence is present in the genome that contains the UAS and a transgene of choice, GAL4 can bind to UAS and drive expression of the transgene that follows the UAS. A TubGAL80^{TS} transgene was added in order to gain temporal control over Kir2.1 expression. At low temperature (18ºC), GAL80^{TS} will be expressed and will prevent GAL4 binding to the UAS sequence, whereas at high temperature (30ºC) GAL80^{TS} will be inactive allowing the expression of any effector gene driven by GAL4. This technique is also known as TARGET (temporal and regional gene expression targeting) (McGuire et al., 2003).

Flies were reared on a standard medium at 18ºC in a 12 hour light / 12 hour dark cycle. Virgin females and virgin males were collected at eclosion under CO_2 anesthesia and then kept singly in vials at 18˚C. In experimental conditions silencing was induced by incubation of flies for ~15 hours at 30˚C, followed by an additional incubation of ~24 hours at 25˚C for acclimatization. Flies under control conditions were always kept at 18˚C and were then joined with the experimental flies at 25˚C 24 hours prior the behavioral experiment. Schematic representations of the different temperature treatments can be visualized in Figure 3.12 Experiments were subsequently performed between 0900h and 1300h at 25˚C and 60% humidity with flies aged for 8-16 days and males aged for 4-8 days (their developmental age was less because of several days spent at 18˚C). Male and female flies were coupled into arenas (16x4, diameter x depth) using an aspirator and then filmed for 30 minutes using a commercial color video camera (Sony Models: HDR-XR520VE, HDR-CX570E, HDR-SR10E).

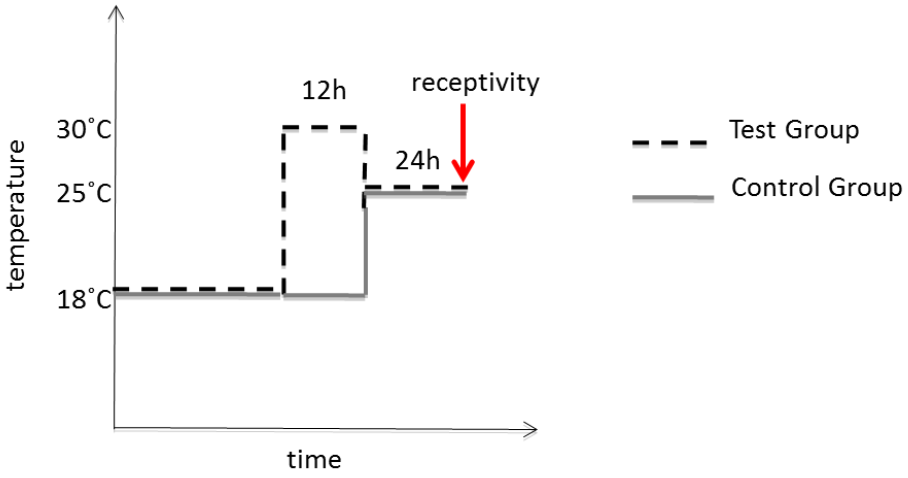

Figure 3.12 Schema of the temperature regime for Kir2.1 expression for receptivity experiments

Maximally 24 arenas were recorded in one film. A light plate was used as light source. The movies were viewed using Sony software ("PlayMemories Home"). Time of arena-placement and time of copulation were scored manually and analyzed with Microsoft Excel 2010 and GraphPad Prism 6.

Wild-type flies of the Canton-S (CS) strain were used to control for the effect of the different temperature treatments on receptivity behavior. No alterations in the mating levels were seen in CS flies that were subjected to the experimental conditions (30ºC) as compared with control conditions. Flies carrying *fru^{M}-GAL4* and *UASKir2.1. TubGAL80^{TS}* transgenes were used to control for the efficiency of neuronal silencing. In experimental conditions they exhibited a reduction to ~5% receptivity. This evidences that our experimental design works.

Staining Protocol

Flies were dissected in PBS (phosphate buffered saline) on a sylgard coated dishes and the brains stored in PBS on ice for up to an hour, using 0.5 mL Eppendorf tubes. Dissected brains and VNCs were fixed by incubation with a 4% paraformaldehyde solution (in PBS) for 30 minutes at RT. The PFA was removed and the fixed tissue incubated in 10% normal goat serum (NGS; in PBS) for 15 minutes and was followed by incubation with antibodies. Antibodies used included: rabbit anti-GFP 1:2000 (v/v) (Invitrogen); mouse anti-bruchpilot (NC82), 1:10 (v/v) (Developmental Studies Hybridoma Bank at the University of Iowa); secondary antibodies were goat anti-rabbit IgG conjugated with Alexa Fluor 488 (1:500 (v/v); Invitrogen), goat anti-mouse IgG conjugated with Alexa Fluor 594 (1:500 (v/v);

Invitrogen) and anti-phalloidin conjugated with Alexa Fluor 594 (1:50 (v/v); Invitrogen).

Primary and secondary antibody incubations varied from 1d to 3d, at 4˚C in agitation. After each incubation brains were washed 3 times for 5 minutes with PBS.

All images were acquired with a Zeiss LSM 710 confocal microscope with a 20x objective (dry) and treated with ImageJ (FIJI).

Brain alignment

For brain alignments we used the CMTK software http://www.nitrc.org/projects/cmtk.

We created a standard brain and a standard VNC using the same software. The VNC we divided into prothoracic-and-mesothoracic and metathoracic-and-abdominal ganglion, or simply upper VNC and lower VNC. This was necessary because the alignment towards the edges of an image decreases in quality and with a 20X magnification large parts of the VNC are very close to the edges. We had about 40 images of each of the parts of the nervous system. Then we aligned each image of the 40 to every other of the same 40 and checked the quality of the alignment by superimposing two images – the aligned one to the one that it was aligned to - in ImageJ (FIJI). The images that compared well to the most other brains were considered as seed brains for the standard creation. The last decision was made subjectively by judging the image for overall quality of alignment of the different areas. Then we created an average, the standard, out of all the images that aligned reasonably well to the seed brain using FIJI Image Expression Parser.

We then went on to align all the images to the standard - now using two channels, the neuropil-marker (NC82) channel and the *GAL4 > UAS-mCD8-GFP* channel.

Proboscis Extension / Bloated Abdomen

To assess the quantity of bloated flies, receptivity videos were viewed and every female counted as bloated whenever it had a constantly extended proboscis. The extended proboscis was more reliably discernible, it was either extended or not, while the degree of bloatedness varied. Flies that had an extended proboscis were always more, or less bloated. Flies that looked bloated occasionally had no extended proboscis.

Egg Laying

After temperature induced activation of Kir2.1-expression (24 hours at 30˚C; control flies at 18˚C), five females (aged 6-14 days; mated the day before) were allowed to lay eggs on apple agar medium covering a 5 cm plate for 24 hours (Figure 3.13).

Counted were only those plates that in the end contained 5 living flies. Data were analyzed using GraphPad software. The statistical test used was the Students t-test.

The number of eggs was calculated as an average: eggs laid per female per experiment.

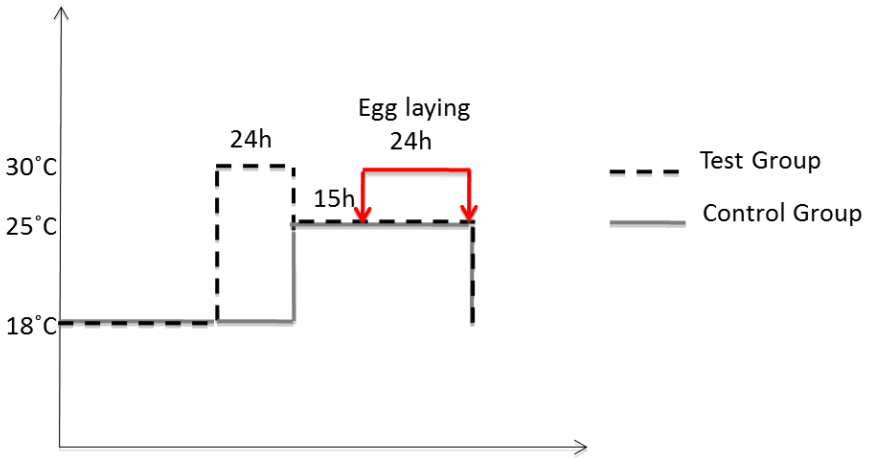

Figure 3.13 Schema of the activation for the egg laying experimente

Statistical Analyses

Receptivity data was statistically analyzed with the Fisher's exact test in Graph Pad 6.

Locomotion, Courtship Index and egg-laying data was statistically analyzed with the student t-test in Graph Pad 6.

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Author Contributions

D.P.H. performed all the experimental work and analyses, with the following exceptions: Image of the posterior brain (Figure 3.8C) was taken and the specimen prepared by Marcia Aranha; Counting of the flies with proboscis extension/bloatedness was performed by Sophie Dias. D.P.H. and M.L.V. designed the experiments.

Competing Financial Interests

The authors declare no competing financial interests.

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IV. Discussion

In this work we presented data that identifies *apterous-GAL4* neurons as critically involved in female receptivity. We provide evidence that the receptivity phenotype is not part of the described sex-peptide sensory circuitry and that it is not part of the postmating switch in general. Using an intersectional approach to reduce the number of neurons we have generated data that further characterizes the *apterous-GAL4* subset of neurons involved in receptivity.

In this Chapter we will discuss our results, put them into context and provide suggestions for further investigation of the circuitry here described.

Introduction

Drosophila melanogaster as a model system for neuroscience is of growing importance. The accessibility of the small nervous system and its malleability by a wide range of tools allows for the elucidation of neural circuits and the unraveling of their architectural logic, on the level of small neural ensembles down to the connectivity of single neurons (Kazama, 2014). Since the early days of *Drosophila* research, courtship has fascinated scientists. It is a tiny spectacle, intriguing because of the stereotypicity of the actions as well as the variability of the sequence of their execution. It is complex in its nature, having two courting parties evaluating each other. And it is as inevitable, or robust, as it is interruptible by genetic perturbation (Hall, 1994).

Silencing *apterous-GAL4* Neurons Reduces Female Receptivity

Which neurons mediate receptivity behavior? Answering this question would allow us to inquire further: What is the information they process? How are they connected and what is their role in yielding a behavioral output?

We investigated the neural substrate that allows females to decide whether to mate with a courting male or not. We aimed at identifying novel neurons involved in receptivity and shine a light on their function in a neural circuit and a behavioral context. In naïve animals the respective circuits are unaltered by experience and comparable from one specimen to the next. We take advantage of an established experimental paradigm – the receptivity assay – and combine it with recent genetic tools to access and manipulate defined sets of neurons.

In a screen of 12 GAL4 driver lines, that were used to express a silencing ion-channel, Kir2.1 in a temporally controlled manner, we uncovered the involvement of *apterous* neurons in controlling receptivity. Silencing *apterous* neurons via the expression of Kir2.1 reduces female receptivity by approximately 50%.

We ascertained various aspects of the nature of *apterous* neurons by performing several control experiments: 1. Does *apterous-GAL4* silencing affect basic, crucial behaviors that influence receptivity? We tested gross locomotion and female attractiveness. The results of both experiments suggest that silencing *apterous* neurons does affect neither locomotion nor female attractiveness. 2. As *apterous-GAL4* intensely labels the optic lobes we asked whether inhibition of vision

by silencing photoreceptor neurons affects receptivity. We found that silencing *GMR-GAL4* labeled neurons does not affect receptivity. 3. Is Apterous protein required in the adult for the control of receptivity? RNAi-mediated knockdown of *apterous* protein only in the adult, allows the tentative conclusion that Apterous is not required for receptivity. In order to strengthen this claim, RT-qPCR (quantitative reverse transcription PCR) could be performed, possibly demonstrating the knockdown of *apterous* transcripts. 4. Does *apterous-GAL4* label neurons that were previously described as mediating receptivity? Examination of the *apterous-GAL4* expression pattern revealed that two areas integrally related to receptivity, are not labeled, the antennal lobe (AL) and the antennal mechanosensory and motor center (AMMC). Additionally we do not see neurons in the uterus that match SP sensory neurons described elsewhere (Häsemeyer, Yapici, Heberlein, & Dickson, 2009; Rezával et al., 2012a; Yang et al., 2009).

Interested in whether silencing *apterous* neurons increases egg-laying – a PMR – we performed an egg-laying experiment. Instead of an increase, which we expected in the case of silencing sex peptide sensory neurons, or no effect, we observed a decrease of egg-laying in virgin as well as in mated females, suggesting that silencing *apterous* neurons does not trigger the postmating switch. We would like to point out that the receptivity reduction that we observe upon neuronal silencing is strong, but not as strong as the one observed when silencing PMR related neurons (Häsemeyer et al., 2009; Rezával et al., 2012a; Yang et al., 2009), indicating a receptivity reduction caused by different neurons.

We have shown that gross locomotion is not affected and the male is properly enticed to court. Yet, it is possible that subtle changes in the

female's behavior lead to altered courtship dynamics and cause receptivity reduction. The females have a bloated abdomen and an extended proboscis, this could make them prone to exhibit a changed locomotion pattern; e.g. they might pause more or less than controls, or they are unable to properly exhibit rejection behaviors due to an inability to extend their ovipositor. A detailed analysis of subtle behaviors could inform this notion. However, when we split the females with silenced *apterous* neurons into two groups, the one not exhibiting bloatedness/proboscis extension still showed strongly reduced receptivity. Furthermore, females with a removed *TH-* or *GAD-GAL80 ∩ apterous-GAL4 > UASKir2.1, TubGAL80^{TS}* intersection still exhibited bloatedness/proboscis extension while they were fully receptive. Both observations strongly indicate that proboscis extension/bloatedness does not cause receptivity reduction.

Intersections of *apterous-GAL4* with GAL80 Lines that Rescue *apterous* Phenotypes

Our next step was to reduce the number of neurons that are silenced within the *apterous* set. We chose two strategies that take advantage of the creation of intersections. This is achieved by the combination of the *apterous* set of neurons with another set of neurons. If there is an overlap, or intersection, between the two sets, the neurons in the intersection will be either excluded from manipulation – by using GAL80 lines – or the neurons in the intersection will be the only ones manipulated - by using the FLIP-OUT technique. The FLIP-OUT technique is more powerful, as the intersection is directly manipulated. The intersection will be silenced or stained and thus stands out. If, on the other hand, the intersection is excluded from

silencing or labeling, via GAL80 expression, the effect of the intersection is only indirectly measured. Unfortunately, we could not use the FLIP-OUT technique as the required transgenes, interacted adversely with *apterous-GAL4*.

We tested several GAL80 lines for rescue effects, in assays for receptivity, egg-laying and proboscis extension/bloatedness, which allowed us to partially separate the roles that different sets of apterous neurons play in mediating these phenotypes.

We found that, when *frulexA > lexAop-GAL80*, *MB247-GAL80*, *Cha-GAL80* or *lk-GAL80* intersect with GAL4-expressing *apterous* neurons, all three tested behaviors were largely rescued to wild-type levels. We also found, however, another type of intersection that mostly rescued receptivity but not the other two behaviors. These intersections were created by *GAD-GAL80* ∩ *apterous-GAL4*, *TH-GAL80* ∩ *apterous-GAL4* and *cry-GAL80* ∩ *apterous-GAL4*. Our results are strong support for the notion that the receptivity reduction through silencing of *apterous* neurons is effected by a specific set of neurons that is separable from the other phenotypes.

Anatomy of *apterous-GAL4* ∩ GAL80 Line Intersections

In a copious experiment using brain alignment we searched for the intersections that rescue wild-type behavior. If images of specimen expressing GFP in all *apterous-GAL4* neurons, aligned to a standard image, are superimposed with images of specimen that do not express GFP in GAL80 expressing neurons, also aligned to the same standard image, differences in expression should be locatable.

However, apart from neurons of the MB and a small area in the SOG we were not able to identify intersections. The intersection in the MB

and the SOG do not directly correlate with rescue of the receptivity phenotype. The fact that we could not identify other areas can be generally attributed to a number of complications: 1. *apterous-GAL4* labels many neurons, often tightly packed. A small intersection in a tightly packed cluster can easily escape detection. 2. Artifactual removal of neurons during dissection. 3. Insufficient resolution for the abundant fine innervations. 4. Possibly rescue can be explained by a level effect, in which a reduction of GAL4 inhibition, would be sufficient for a rescue. Reliably controlling for levels of intensity of labeling to this degree is not possible as transgene levels vary with the age of the fly and with the temperature it is kept at. Further variation can occur during staining.

Recently, colleagues in the laboratory silenced *aperous-GAL4* neurons only in the brain using *OTD-FLP*. This resulted in receptivity reduction strongly suggesting that neurons located in the brain are responsible for the receptivity phenotype. So, where are they? In the schemata in Chapter III we have represented the observed clusters of *apterous* neurons (Figure 3.8B and D). Cells in these clusters must be responsible. With this new knowledge it might be possible to focus on the brain clusters and identify the crucial neurons. As most *apterous* brain neurons are located posteriorly it will be helpful to image the brain not only from one side, as in this study from anterior to posterior, but also from posterior to anterior. This will ensure optimal imaging of the fluorescence at the posterior brain. Moreover, image resolution can be increased by increasing pixel density and by using not only 20x magnification, but 40x or even 63x. This will require creation of new standards with respect to the area in question. The use of anti-bodies against TH and GAD might help to visualize

areas of co-staining with *apterous-GAL4* > *UAS-mCD8-GFP* and further inform anatomical pursuits.

Mushroom Body and *leucokinin* Neurons

We observed a full rescue of receptivity with the line *MB247-GAL80* implying that neurons in the MB express Kir2.1 and cause receptivity reduction. In addition we note differences in expression in the MB with several lines. This is strong support for the intriguing hypothesis that MB neurons are involved in receptivity. As a memory and learning center and recently described also as a decision making center (DasGupta, Ferreira, & Miesenböck, 2014), the MB could easily be conceived as the place where courtship stimuli converge, where they are processed and accumulated until a threshold is reached and the decision for mate acceptance is signaled. This hypothesis however has been refuted by two studies (Fleischmann & Cotton, 2001; Kido & Ito, 2002) that observed no receptivity reduction after hydroxyurea ablation of the MB. Nonetheless, silencing neurons with Kir2.1 is very different from ablating them and taken together with our evidence we decided that it is worthwhile to test whether Kir2.1-mediated silencing of the MB reduces receptivity. However, when we silenced neurons labeled by *OK107-GAL4*, a line that is described to label most MB neurons (Aso et al., 2009), we did not observe receptivity reduction. In order to test the effect of directly silencing *apterous* neurons in the MB, we silenced neurons in the intersection *MB247-FLP* ∩ *apterous-GAL4*. However, we did not observe receptivity reduction. Another explanation is provided by the observation by us and others (internal communication) that GAL80-lines can have a broader expression pattern than their GAL4 counterpart (Rezával et al. 2012).

lk-GAL80 was a second line with a restricted expression pattern that yielded rescues of the three phenotypes and exhibited differences in expression in the MB. After anatomical examination we found an additional area in which we observed a possible difference in expression. It is located in the superior third of the SOG. In females only expressing *apterous-GAL4* and *mCD8-GFP* a triangular area was intensely labeled. We called it *apterous* SOG delta, ASD. In females expressing *lk-GAL80* only the superior triangular shape was still discernible, while the inferior band disappeared. The SOG was directly implied in female receptivity by Sakurai et al. (2013). Yet, when we used *lk-GAL4* to silence the respective neurons we did not observe a reduction in receptivity.

These two results surprised us and led us to speculate about the existence of two redundant pathways; one defined by *lk-GAL4* the other by *OK107-GAL4* (and by *MB247-GAL80*). When we tested this hypothesis we also obtained negative results: Simultaneous silencing of neurons labeled by *OK107-GAL4* and *lk-GAL4* did not have an effect on receptivity levels. This is potentially explained by differences in the strength of different driver lines; both GAL4 lines may not yield sufficient Kir2.1 expression to adequately silence the neurons. Yet, this explanation is unsatisfactory as *lk-GAL4* driven Kir2.1-expression is sufficient to yield proboscis extension/bloatedness and GFP expression with either line shows strong labeling of neurons.

apterous-GAL4 and PMRs

Most of what we know about neural networks involved in receptivity pertains to the PMRs. When SP reaches the female reproductive tract it will bind to its cognate receptor that is expressed in described SP

sensory neurons, which then signal the presence of sperm downstream, effecting reduced receptivity and increased egg-laying.

As our results suggest *apterous* neurons do not intersect with these primary sensory neurons: silencing *apterous* neurons reduces receptivity (which is consistent with *apterous* neurons being SP sensory neurons) but does not increase egg-laying (which is not), to the contrary, it decreases egg-laying in the virgin as well as in the mated female. As mentioned before, the receptivity reduction that we observe upon neuronal silencing is strong, but not as strong as the reduction observed when silencing SP sensory neurons (Häsemeyer et al., 2009; Rezával et al., 2012a; Yang et al., 2009). Moreover we do not observe *apterous* neurons in the reproductive tract or the abdominal ganglion, that would match the SP- neurons described in these publications. Finally, *ppk-GAL80* that intersects with SP sensory neurons, does not rescue receptivity.

Several scenarios are consistent with these observations. 1. *apterous* neurons mediate virgin receptivity. 2. The pathway signaling PMRs bifurcates at some point - one path signaling reduced receptivity, the other increased egg-laying; *apterous* neurons would only intersect with the receptivity path. 3. *apterous* neurons do in fact intersect with neurons in early steps of the pathway inducing both PMRs, reduced receptivity and increased egg-laying, but we do not see increased egg-laying because of pleiotropic effects of *apterous* silencing: some unidentified silenced *apterous* neurons elicit behaviors or neuronal responses that counteract the ones signaling increased egg-laying. For instance the phenotype of extended proboscis/bloatedness may not allow for eggs to be laid.

In this context we have to discuss findings by Soller et al. (2006). They claim that *apterous* neurons indeed signal PMRs via neurons in the VNC. However their results are difficult to compare to ours: Firstly, they inject females with SP, which results in females behaving as mated. Secondly, instead of a single pair courtship assay they use a group assay with three females and six males in a vial for one hour. Moreover they perform it at 29˚C in order to boost expression levels of TNT. Then, when silencing *apterous-GAL4* neurons with TNT, they observe a ~45% increase in receptivity compared to controls not expressing TNT and argue that activity in *apterous* neurons is required to reduce receptivity in response to SP.

Taken together we think it is most likely that *apterous-GAL4* either labels neurons in the brain that process aspects of female receptivity which do not pertain to PMRs or that they label brain neurons at some point after a hypothetical bifurcation of PMR signals.

Future Experiments

The availability of lines that target specific neurons is a serious impediment in manipulating desired sets of neurons and performing the most efficient experiments – a problem we encountered throughout this work. The recently developed method CRISPR allows the fast, targeted insertion of transgenes into the genome. Thus, in theory it should be feasible to create a variety of useful lines. For instance new *apterous-GAL4* lines could help avoid the problems encountered with the combination of *apterous-GAL4* with *fru-FLP*. As Apterous is a target of Fruitless combinations involving these two sets of neurons will be essential (Neville et al., 2014). But lines required for other techniques can be more readily acquired too. The use of split-GAL4 lines for example provides an alternative means for

targeting subsets of driver lines. The intersection here is created by two lines overlapping, each expressing one part of the GAL4 molecule. Reconstitution of a competent GAL4 molecule in the intersection then allows for regular binding to UAS, yet confined to cells within the intersection (Gratz, Wildonger, Harrison, & O'Connor-Giles, 2013; Gratz, Cummings, et al., 2013; Port, Chen, Lee, & Bullock, 2014).

Once the essential *apterous-GAL4* neurons are identified an approach to identify synaptic partners, GRASP (GFP Reconstitution Across Synaptic Partners), can be applied. This technique uses split versions of GFP. One part can be designed to express in axons, the other in dendrites, if two sets of neurons are connected, a competent GFP molecule will be reconstituted across the synaptic cleft. This can be helpful in elucidating connectivity of different sets of neurons. Another approach involving photoactivatable GFP (PA-GFP) and 2-photon microscopy may be applied to trace neurons and their synaptic partners (Datta et al., 2008).

Instead of silencing neurons and testing for changes in behavior, labeling neurons with a calcium indicator and testing for neural activity in response to the presentation of stimuli can be useful. Calcium imaging refers to the optical recording of neural activity. A genetically encoded calcium indicator (GECI) is expressed in neurons. Most neurons, in the moment of excitation, get flooded with calcium, which will bind to the GECI molecule that changes its conformation in response and becomes fluorescent. Thus a live fly brain can be imaged while it is exposed for example to sound or odor, and the response of neurons, if there is one, can be recorded. Calcium imaging may not be only useful in identifying the neurons

that do respond, but also understanding to what they respond (Venken, Simpson, & Bellen, 2011).

Lastly, it will be crucial to have a profound mechanistic understanding of the female courtship behavior in order to relate it to the neuronal circuitry that brought it about. This could be achieved by a sophisticated automated analysis of high quality videos of behaving couples.

Conclusions

The study presents data that strongly implies *apterous* neurons in control of virgin female receptivity. We expect the subset of *apterous* neurons required for this behavior to be novel and small. Their neural identity is likely to be characterized by being *fru*, *cha*, *lk*, *cry*, *TH* and *GAD* positive. These results represent firm ground on which to launch follow-up studies that potentially will lead to an understanding of the neuronal underpinnings that mediate virgin female receptivity behavior in *Drosophila melanogaster.*

Summary of Future Work

1. Create new lines using CRISPR that would allow testing of more intersections with FLP-lines and split-GAL4 lines and that moreover would help to avoid the problems encountered in this work.
2. Concentrate brain alignment and anatomical efforts on *apterous-GAL4* brain clusters.
3. Use calcium imaging to elucidate relevant stimuli.
4. Elucidate connectivity of essential *apterous* receptivity neurons to other neurons.
5. Detailed analysis of female courtship behaviors.

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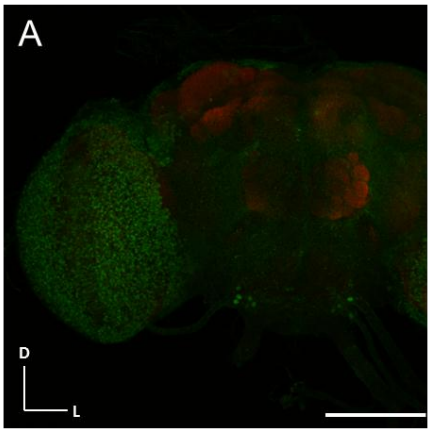
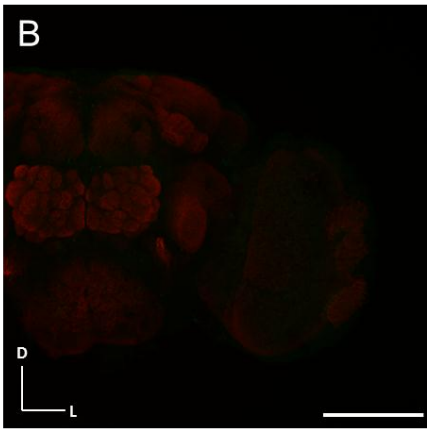
Appendix A

Representative z-projections of whole stacks imaged at 20x, scale bar: 100µm. Image A shows a brain of a temperature control fly – the Apterous-GFP fluorescence without induction of knockdown. Image B shows the brain of a fly in which *apterous* knockdown was induced for four days at 30ºC – only faint traces of Apterous-GFP fluorescence remain.





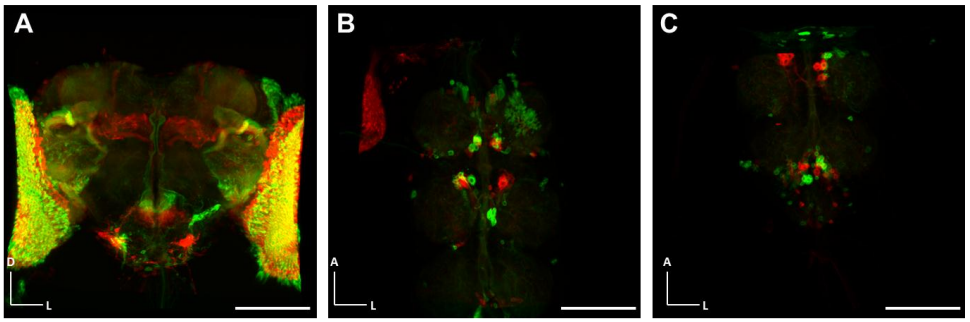
Appendix B

Photography of two flies with *apterous-GAL4 > UAS-Kir2.1, Tub-GAL80^{TS};* the temperature control is on the left. The test fly on the right in comparison shows a strongly bloated abdomen and a constantly extended proboscis.

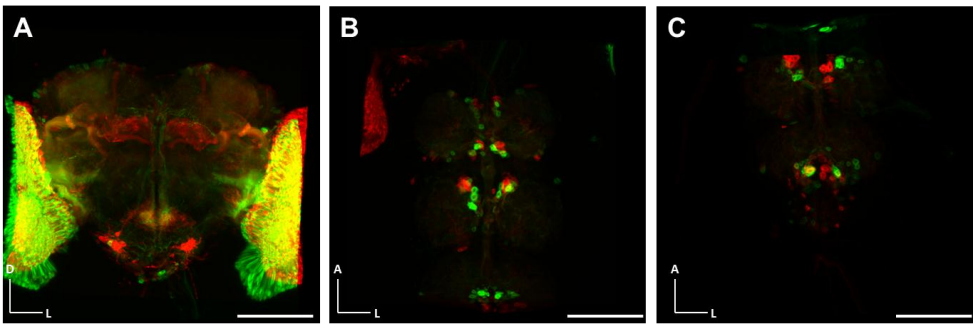
Appendix C

Representative z-projections of whole stacks imaged at 20x, scale bar: 100µm; aligned to the standards shown in Chapter III. Neurons of flies with *apterous-GAL4* > *UAS-mCD8-GFP* are shown in red, superimposed with neurons of flies that additionally carry a GAL80 insertion, shown in green. Image A shows the brain, B the pro- and mesothoracic ganglion and Image C shows the metathoracic and abdominal ganglion.

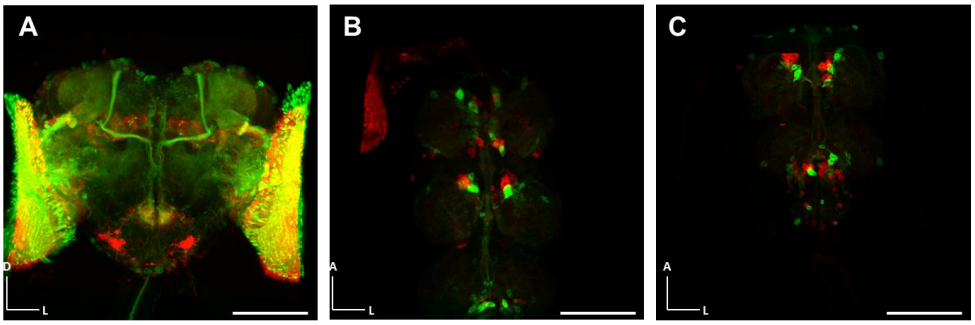
MB-GAL80



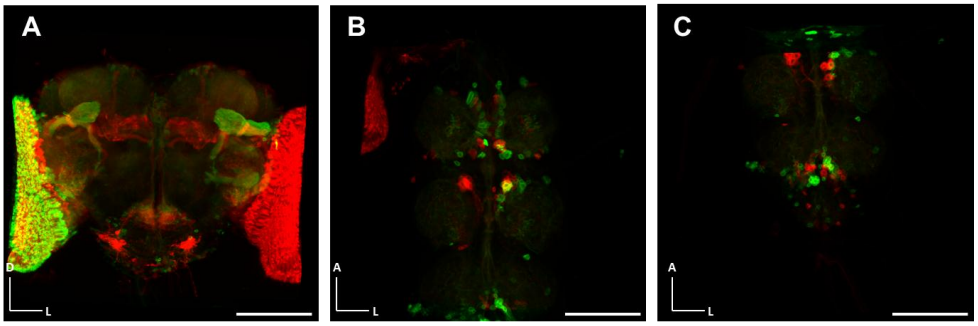
frulexA > lexAop-GAL80



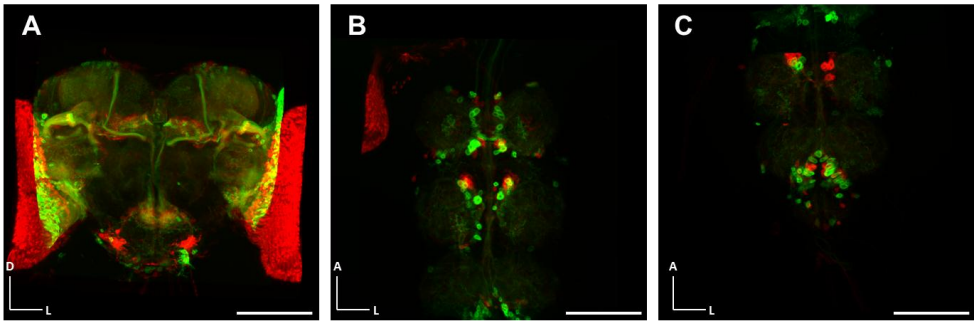
Cha-GAL80



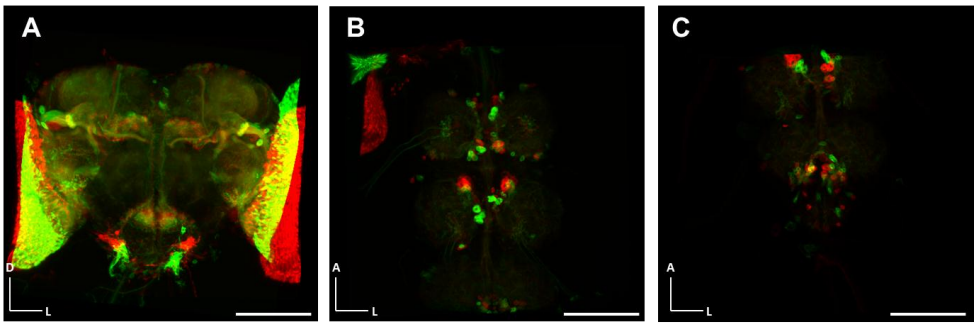
lk-GAL80



TH-GAL80



GAD-GAL80



cry-GAL80